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(57) Abstract

A promintron sequence derived from an intervening sequence of the *rolA* gene of *Agrobacterium rhizogenes* strain A4 is described. The sequence is able to drive gene expression within bacteroids in all stages of nodule development in order to obtain, over the developmental time of the nodule, a constitutive expression of the gene(s) of interest. Uses of said sequence, derived vectors and recombinant bacteria are also described.

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METHOD TO CONTROL GENE EXPRESSION IN BACTERIA, NAMELY *RHIZOBIACEAE*, TO IMPROVE ROOT NODULE DEVELOPMENT, NITROGEN FIXATION AND PLANT BIOMASS PRODUCTION.

DESCRIPTION

The present invention relates to the use of the promintron DNA sequence of the *rolA* gene of *Agrobacterium rhizogenes* strain A4, or of promintron DNA sequences derived thereof, or of promintron DNA sequences of homologous genes from other strains of *Agrobacterium rhizogenes*, for promoting gene expression at the transcriptional level in recombinant bacteria and to achieve, for example, synthesis of phytohormones within bacteria and/or bacteroids.

The present invention demonstrates that the *iaaM* gene of *Pseudomonas syringae* and the *tms2* gene of *Agrobacterium tumefaciens* are able to improve the nodulation process and nitrogen fixation when expressed as bicistronic unit in *Rhizobia* under the control of the promintron sequence. The promintron is particularly suited and useful for the use to drive gene expression within bacteroids in all stages of nodule development in order to obtain, over the developmental time of the nodule, a constitutive expression of the gene(s) of interest. Therefore, constitutive here means that, by different expression patterns at various stages of nodule development, a gene expression in all parts of the nodule where plant cells harbour *Rhizobia* is achieved.

The invention stems from the undisclosed demonstration that the 85 bp intron sequence of the *rolA* gene of *Agrobacteria* strain A4 is able to promote gene expression in prokaryotic cells. Therefore, the 85 bp DNA sequence has been named "promintron", indicating its function of intron in eucaryotic cells (Magrelli, A. et al. (1994) Science, 266, 1986-1988), and of promoter in prokaryotic cells. The term "promoter" refers to

nucleotide sequences necessary for transcription initiation, i.e. RNA polymerase binding, and also includes for example the -35 and -10 boxes, or other regulatory regions.

The promoter activity in *Rhizobia* of the *rolA* promintron resulted to be able to get gene expression in a novel and peculiar pattern within root nodules, resulting in a constitutive expression.

The invention is particularly useful: i) to alter nodule development and to increase nitrogen fixation by either nodulating plants and/or by nitrogen fixing bacteria; ii) to improve plant growth and to accelerate plant biomass production by inoculating leguminous plants with genetically modified *Rhizobia* (*Rhizobia* is here used as a collective name including *Rhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Bradyrhizobium*).

The present invention also relates to DNA constructs in which said promintron controls the expression of a DNA sequence, which upon expression leads to the above mentioned effects. Furthermore, the present invention relates to genetically engineered bacteria which can improve nitrogen fixation and consequently, plant biomass production. Furthermore, the improvement of nitrogen fixation in plants might be achieved in the presence of concentrations of nitrate negatively affecting the nodulation process.

The world population will most likely increase of 2 billions people in the next 25 years mostly in Asia and Africa. Proteins coming from legumes represent actually 33% of total human diet. Legume plants are able to self provide nitrogen through associations with nitrogen fixing bacteria of the genus *Rhizobia*, i.e *Rhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Bradyrhizobium*. Other plants do exist not belonging to the *Leguminosae*, and yet able to host bacteria in a nitrogen-fixing nodule. A plant not belonging to the

leguminous family interacting with nitrogen fixing *Rhizobia* is *Parasponia* (Ulmaceae). Furthermore, actinomycetes of the genus *Frankia* interact with plant species from eight different families and cause the production of root nodules (Pawlowski, K. et al. (1998) in: Biological Nitrogen Fixation for the 21st Century, eds. C. Elmerich, A. Kondorosi and W.E. Newton, Kluwer Academic publisher, pp.199-201). Symbiotic nitrogen fixation plays a major role in the nitrogen cycle. Nevertheless, agriculture exploitation results in a large uptake of organic nitrogen from the soil, which is compensated by providing plants with fertilisers. Nitrogen fertilisation however, provokes severe environmental problems because its excess contributes to water pollution. Nitrogen fertilisation represents one of the most important part of the farming costs. The world consumption of nitrogen fertilisers increased in the last 55 years from 3 to 100 TG ($1TG=10^{12}g$). To produce nitrogen fertilisers, all over the world 2 to 5% of the not renewable energy sources (e.g. oil and other forms of fuel) are burned every year. An environmental problem associated with the use of synthetic nitrogen fertilisers is leakage of excess nitrate into the underground water. Up to 10% of the released nitrate ends as nitrous oxide, a greenhouse gas whose energy reflectivity amounts to 180 times that of carbon dioxide (Hardy, R.W.F. and Eaglesham, A.R.J. (1995). In: Nitrogen Fixation: Fundamentals and applications. I.A. Tikhonovich et al., (eds.), pp. 619-620). Many large towns in industrialised countries are presently facing the problem of nitrate pollution of drinkable water, and water price for human use will rapidly increase.

Therefore, the general problem underlying the present invention is represented by the need to improve the cultivation of leguminous and other nodulating plants, with the aim to contribute to a drastic decrease

of the nitrate release in the environment. A solution to these problems is either to increase the cultivation of plants able to fix atmospheric nitrogen or to increase their nitrogen fixation capacity.

Biological Nitrogen Fixation (BNF) by free-living, associative and symbiotic nitrogen-fixing microbes is responsible for the conversion of about 120 million tons of atmospheric nitrogen into ammonia every year, which represent 40% of the total nitrogen request (Vance, C.P., Third European Nitrogen Fixation Conference, Lunteren, The Netherlands, 1998, p. 17); symbiotic nitrogen fixation is the major naturally occurring mechanism by which nitrogen is reduced (Franssen, H.J. *et al.* (1992) Plant Molec. Biol., 19, 89-107). Members of the *Rhizobiaceae* family, which includes nitrogen-fixing *Rhizobia*, are able to enter into symbiosis with mainly leguminous plants, resulting in the formation of root nodules. Within the cells of the root nodule, the bacteria differentiate in bacteroids. Mature bacteroids fix atmospheric nitrogen and convert it into ammonia by a nitrogenase activity. The so-produced ammonium is exported from the nodules and is incorporated into plant amino acids, ureids and azotated bases. Thus, in the presence of a nodulated root, plant growth is independent of alternative nitrogen sources.

Root nodule development is the result of a symbiotic interaction controlled by several factors. Early phases of this interaction require the exchange of chemical signals between the two partners: the plant root and the bacteria. The plant root produces chemical signals, such as flavonoids, which attract the *Rhizobia*, and most importantly do induce the expression of bacterial genes, the *nod* genes, involved in the production of the so-called Nod factors. The Nod factors are lipooligosaccarides which play a crucial role in the early phases of nodule development (Denariè, J., Debellè,

F., and Promè, J.-C. (1996) Annual Review Biochemistry 65, 503-535).

While the role of the Nod factors in triggering root nodule development is well established, the role of phytohormones in root nodule development is still unclear. Phytohormones are plant growth factors able to modify plant growth and development. Since phytohormones are involved in almost any developmental and/or physiological process of the plant, a role of phytohormones in nodule development is most reasonable. However, despite efforts aimed to address the general question of a role of phytohormones in nodulation, in 1994 Hirsch and Fang summarised the state of the art with the following words: "Important questions remains unanswered: *which* hormones take part in nodule formation, and *how* are they involved?" (A.M. Hirsch and Y. Fang, (1994) Plant Mol. Biol. 26, 5-9).

Already in 1936, Thimann (Thimann, K. (1936) P.N.A.S. USA, 22, 511-514) proposed that auxin(s) plays a role in nodule development, and that root nodules do contain auxin(s). Furthermore, based on biological assays, Thimann showed that the auxin content, estimated as auxinic biological activity, within a nodule is higher than that one of uninfected roots.

Nowadays, it is well established that free living *Rhizobia* do contain auxins, such as indole-3-acetic acid (IAA), indole-3-ethanol, indole-3-aldehyde and indole-3-methanol (Ernstsen, A. et al. (1987) Planta 171, 422-428). Furthermore, flavonoids, i.e. chemical signals produced by the plant root which attract *Rhizobia* and stimulate the synthesis of Nod factors, stimulate IAA synthesis in *Rhizobia* grown in liquid culture (Prinsen, E. et al. (1991) FEBS Letters, 282, 53-55).

Although these data are consistent with a possible role of auxin(s) in nodule development, Hirsch in 1992 reported that "*Rhizobia* can synthesize plant hormones,

but mutations in their auxin-producing genes do not prevent nodulation, nor do Nod- mutants fail to produce hormones (Hirsch, A.M. (1992) New Phytologist, 122, 211-237).

Other studies addressing the "plant contribution" to the nodulation process, such as those of Hirsch, A.M. et al. (P.N.A.S. USA (1989) 86, 1244-1248), of Allen, E.K., Allen, O.N. and Newman, A.S. (Am. J. Botany (1953), 40, 429-435), and of Torrey, J.G. (in: "New root formation in plants and cuttings", 1986 ed. Jackson MB, Martinus Nijhoff Publishers, Dordrecht, 31-66), have found that inhibitors of polar auxin transport induced pseudonodules on alfaalfa roots. Mathesius et al. (The Plant Journal (1998) 14, 23-34) has recently shown that auxin transport inhibition precedes root nodule formation. Kijne, J.W. et al. (Third European Nitrogen fixation Conference, Lunteren, The Netherlands, 1998, p. 24), have shown that: i) a local increase of IAA precedes induction of cell divisions in the inner cortex and ii) nitrate inhibits the response to IAA rather than its accumulation.

Further indications of a role of auxinic phytohormones produced by the plant tissue in nodule development come from variants of *Medicago varia*, defined as auxin sensitive and usually containing a higher content of auxins, which formed more nodules than less sensitive variants (Kondorosi, E. et al. (1993) In: Nester, E.W. and Verma, D.P.S. eds., Advances in Molecular-Genetics of Plant microbe interactions, pp. 143-150). Furthermore, plants transgenic for the *rolB* gene nodulated faster and had more nodules (Kondorosi et al., ibidem). RolB is known to increase auxin sensitivity. The function of rolB is considered by some authors not to affect auxin content and to be a membrane receptor for the auxin IAA, whereas others (PCT Application No. WO 98/28430) consider the RolB protein a

beta-glucosidase able to release the auxin indolethanol from indolethanol-glucoside. Indolethanol is considered to be an auxin per se, and it is converted to IAA, the major form of auxin in plants (Sembdner, G. et al. (1980) in: Encyclopedia of Plant Physiology, vol. 9: Hormonal regulation of development. I. Molecular aspects of plant hormones, pp. 281-444; MacMillan, J. Ed., Springer, Berlin, Heidelberg, New York; Sandberg, G., Crozier, A., Ernstsen, A. (1987) In: The Principles and practices of plant hormone analysis, vol. 2, pp. 169-301, Rivier, L., Crozier, A. eds. Academic Press, London).

The present invention has addressed three aspects related to the aforementioned problems: i) is it possible to modify root nodule development by expressing auxin synthesising genes within *Rhizobia*?; ii) has the plant nodulated by the genetically modified strain of *Rhizobia* an increased capacity to reduce atmospheric nitrogen to ammonia? iii) is the plant nodulated by the genetically modified *Rhizobia* altered in its growth, and is such alteration improving plant biomass production?

Genes coding for enzymes able to modify auxin metabolism can be taken from the genomes of plants, but also from the genomes of bacteria (Spena, A., Estruch, J.J. and Schell, J. (1992) Current Opinion in Biotechnology 3, 159-163). Whatever the origin of the gene(s) and/or coding region(s), the proper specificity of expression within the bacteria and, most importantly, within the bacteroids must be conferred to the said coding region(s) by positioning the coding region(s) under the control of a proper promoter (DNA) sequence.

The present invention solves the problems of the prior art by providing technical means to improve gene expression in recombinant bacteria, preferably *Rhizobiaceae*, and most preferably *Rhizobium*, and it is easily utilised to properly control the expression of genes able to increase the auxin content and, as

consequence, to improve the nodulation and nitrogen fixation processes. The promintron sequence of the invention is able to promote prokaryotic expression in all the zones of the nodule where bacteroids are located with a temporally defined pattern of expression. Furthermore, the promintron drives gene expression during the exponential phase of growth and it is induced during the stationary phase of growth of free living bacteria. All these features are very advantageous when gene expression is to be promoted in nitrogen fixing soil bacteria under both exponential and stationary phase of growth, and in bacteroids within the root nodule.

In particular a gene construct of the invention comprises the promintron sequence of the *rolA* gene, as in SEQ ID No.1, and the coding regions of the *iaaM* from *Pseudomonas syringae* subsp. *savastanoi* and the *tms2* gene from *Agrobacterium tumefaciens*. The two coding regions have been built as bicistronic unit under the control of the *rolA* promintron, whose promoter activity takes place in the nodule in an undisclosed and novel pattern of expression. The combination of the promintron to the bicistronic unit, and its introduction in *Rhizobia*, has allowed the authors of the invention to show that auxin affects nodule development and function. The use of the two coding regions is meant to increase the synthesis and content of IAA, and consequently to increase also the synthesis and content of IAA conjugates (collective name indicating all the forms of IAA covalently conjugated either to amino acids or to glucose or to other chemical moieties) in the plant cells of the root nodule. The same effect might be achieved either by using the *iaaM* and *tms2* genes from other *Agrobacteria* or *Pseudomonas* strains, or by using other genes able to increase IAA content.

Since *Rhizobia* usually do not contain indoleacetamide, neither are able to convert tryptophane

to indoleacetamide (IAM), nor are able to convert IAM to IAA (Ernstsen et al., 1987, ibidem), the authors have engineered a novel biochemical pathway which requires both genes to efficiently synthesise IAA from tryptophane. However, in those *Rhizobia*, such as *Bradyrhizobium*, which do contain the *bam* gene - an endogenous homologue of the *tms2* gene (Sekine, M., Watanabe, K. and Syono, K. (1989) J. Bacteriol. 171, 1718-1724) - this might be sufficient to express only the *iaaM* gene under the control of the promintron DNA sequence. A functional homologous of *tms2* is also present in some higher plants as shown in the PCT Application WO98/28430.

In a further embodiment of the present invention the DNA sequence expression of which is driven by the promintron is one coding for indolepyruvate decarboxylase. Indolepyruvate decarboxilase is the key enzyme in the indolepyruvic pathway of IAA synthesis in which IAA is synthesised from tryptophane. The conversion of indolepyruvic acid to IAA is the rate limiting step of this pathway. A DNA sequence encoding a suitable indolepyruvate decarboxylase has been cloned, for example, from *Enterobacter cloacae* (see, e.g. Koga et al., Mol. Gen.Genet. (1991), 226, 10-16).

The term "auxin" comprises in this context naturally occurring and synthetic organic substances acting as phytohormones in the sense that they promote elongation of shoots and inhibit elongation of roots, preferably in low concentrations, most preferably already in concentrations lower than 10^{-6} M. Preferably, an auxin shows at least one of the following effects on plant development: stimulation of cell division, of cell elongation, and/or of cell expansion, of apical dominance, stimulation of xylem differentiation, stimulation of the cell elongation and cell division activity of the cells of the cambium, stimulation of

lateral and adventitious root formation, stimulation of nodulation, of germination, of leaf epinasty, of ovary cell growth, of parthenocarpy, of the formation of female flowers and of leaf expansion. More particularly, the term "auxin" refers to indole 3-acetic acid (IAA) which is most likely synthesised in plant cells from tryptophane via indole-3-pyruvate and indole-3-acetaldehyde, and which is degraded via enzymatically catalysed oxidation. However, the term also comprises other naturally occurring compounds which act as an auxin and which are derived from indole or another compound, for example, the naturally occurring phenyl acetic acid which is a non-indolic auxin or 4-(indole-3-yl) butyrric acid. Furthermore, this term comprises compounds from organisms other than plants or chemically synthesised compounds which have at least one of the effects on plant development as listed above. An example for such compound is (2,4-dichlorophenoxy)-acetic acid (2,4-D).

In a preferred embodiment the DNA sequence linked to the promintron codes for a polypeptide which is naturally involved in the biosynthesis of at least one auxin. The expression of the DNA sequence in *Rhizobia* cells leads to an increase in the biological (for example, enzymatic) activity of this polypeptide and consequently to the increase of the content of at least one auxin first in the bacteroid, and after auxin export from the bacteroids, in plant cells of the nodule. Thus, in principle, by this embodiment it is contemplated that the auxin content and/or activity can be increased by increasing the biosynthesis of at least one auxin due to a stimulation/acceleration of a biosynthetic pathway leading to the synthesis of auxin. In another preferred embodiment the DNA sequence linked to the promintron codes for a protein which is naturally not expressed in *Rhizobia* and which upon expression in bacteroids leads to the synthesis of at least one auxin or a precursor of an

auxin from a metabolite present in the bacterial cell and/or bacteroid. One example of a gene which is preferably used for the purpose of the present invention is the *iaaM* gene of *Pseudomonas syringae* subsp. *Savastanoi*, the ethiological agent of plant tumors in olive and oleander trees (Spena, A., Estruch, J.J., Schell, J., Curr. Opinion in Biotechnology (1992), 3, 159-163). The neoplastic development is caused by phytohormones synthesised by the bacteria, which are then secreted into the surrounding tissues and stimulate the localised growth of plant cells. Among the genes involved in the pathogenesis of this type of tumour, the *iaaM* gene codes for the indoleacetamide monooxigenase, and it is responsible for converting by oxidation the amino acid tryptophane to indoleacetamide. Indoleacetamide has no particular auxin activity, but it is converted to IAA by the hydrolase encoded by the *iaaH* gene of *Pseudomonas syringae* subsp. *Savastanoi*, which is homologue to the *tms2* gene of *Agrobacterium tumefaciens*. In plant tissues, indoleacetamide is also converted to IAA, either chemically or by unspecific hydrolases.

The *iaaM* gene from *Pseudomonas syringae* subsp. *Savastanoi* is known and its sequence has been published (Yamada et al. (1985) P.N.A.S. USA, 82, 6522-6526). The *tms2* gene from *Agrobacterium tumefaciens* is known and its sequence has been published (Klee, H. et al. (1984) P.N.A.S. USA 81, 1728-1732). According to the invention genes homologous in function to the *iaaM* gene of *P. syringae* and to the *tms2* gene of *Agrobacterium tumefaciens* might be used for the purpose of this invention. Such genes which are preferably also homologous with respect to the nucleotide sequence can be isolated by the person skilled in the art using known methods, e.g. the screening of cDNA or genomic libraries with probes designed on the basis of the *iaaM* gene of *Pseudomonas syringae* and/or *tms2* gene of *Agrobacterium*

tumefaciens. Such genes with an activity similar to that of the *iaaM* gene product of *P. syringae* have been cloned, for instance, from some strains of *Agrobacteria* (i.e. *Agrobacterium tumefaciens* and *rhizogenes*; see, for instance: Klee et al. (1987), Gene Dev, 1,186-196; White et al. (1985) J. Bacteriol., 164, 33-44; Cardarelli et al. (1987), Mol. Gen. Genet., 208, 457-463.

The 85 bp DNA promintron sequence of the *rolA* gene from Ri plasmid A4 (SEQ ID No.1) promotes gene expression of DNA sequences positioned under its control (i.e. linked to its 3' end) in prokaryotic systems, such as *E.coli*, *Rhizobia* and *Agrobacteria*, during exponential, post-exponential and stationary phases of growth. Furthermore, the promintron promotes gene expression in bacteroids within root nodules. The person skilled in the art may easily and with no inventive effort isolate promintron sequences from other strains of *Agrobacterium rhizogenes* and/or isolate functional homologue promoters, namely promoters driving gene expression with an identical pattern in bacteroids within root nodules and/or in free living bacteria.

It is therefore a specific object of the present invention the use of the promintron sequence of the *rolA* gene from *Agrobacterium rhizogenes* as in SEQ ID No. 1, or of DNA sequences comprising said promintron sequence, or of functional homologue or portion thereof, to induce the expression of a DNA coding sequence, in recombinant bacteria during exponential, post-exponential and stationary phase of growth, and in bacteroids within root nodules, said coding DNA sequence being under the control of said promintron sequence, or of functional homologous promoters. Preferably the recombinant bacteria belong to either the *Enterobacteriaceae* or the *Rhizobiaceae* families, more preferably are *E. coli*, *Rhizobia* or *Agrobacteria*.

In a preferred embodiment the recombinant bacteria are of the *Rhizobia* genus, either within symbiotic root nodules or in a free living status. When bacteria are within symbiotic root nodules, they are either bacteroids of stage I, II, III, IV, V, or *Rhizobia* present in the apoplastic space, or *Rhizobia* present in the senescence zone, or *Rhizobia* present in the nitrogen fixing zone, or *Rhizobia* present in the invasion zone.

It is further object of the invention a recombinant DNA molecule comprising the promintron sequence according to the invention, or functional homologue or portion thereof, and covalently linked to the 3' end of the promintron sequence, a DNA coding sequence, wherein the recombinant DNA molecule is either harboured by episomal elements or integrated in the bacterial genome. Preferably the DNA coding sequence is either a monocistronic or a polycistronic transcription unit, more preferably encodes a protein involved in plant hormone synthesis and/or metabolism, most preferably encodes a protein involved in plant hormone auxin synthesis and/or metabolism, most preferably encodes a protein involved in the synthesis and/or metabolism of the auxin IAA or of the auxin indolethanol, even more preferably encodes the iaaM protein from *P. syringae* subsp. *savastanoi* or an homologous (i.e. with a similar enzymatic activity) thereof and/or encodes the tms2 protein from *A. tumefaciens* or an homologous thereof. In a preferred aspect the recombinant DNA molecule according to the invention comprise both the *iaaM* and the *tms2* coding regions.

Alternatively the recombinant DNA molecule of the invention comprises a DNA coding sequence encoding the indolepyruvate decarboxylase from *Enterobacter cloacae* or an homologous thereof.

It is a further object of the invention genetically engineered bacteria comprising the recombinant DNA

molecule described. Bacteria are preferably comprised in the following list: *Rhizobia*, as above specified, *Azotobacter, Azospirillum, Anabaena, Enterobacteriaceae*.

It is in the scope of the invention the use of the recombinant DNA molecule to increase nodule size and/or activity, and/or to increase plant biomass production.

It is in the scope of the invention the use of the recombinant DNA molecule to increase the capacity of nitrogen fixation of root nodules.

The invention will be described in some examples by reference to the following figures:

Figure 1: Schematic drawing of the chimeric reporter genes. The 86AGUS construct contains: i) an 86 bp long DNA fragment comprising the 85 bp long promintron sequence as shown in SEQ ID No. 1 plus one A residue at its 3'end; ii) the DNA sequence corresponding to the first 40 amino acids of the rolA protein fused to iii) the *uidA* coding region. The 85-17GUS construct contains 5' to 3' end: an Eco RI restriction site (GAATTC), the promintron sequence of SEQ ID No. 1, a 17 bp linker sequence (SEQ ID No. 2), a KpnI restriction site (GGTACC), before the ATG of the *uidA* coding region. Δ GUS indicates the construct containing just the *uidA* coding region, i.e. without any promintron sequence. Promintron indicates the 86 bp sequence comprising the 85 bp of the *rolA* promintron (from -1 to -86 being +1 the A of the ATG initiation codon of the *rolA* gene). *RolA* coding region indicates the sequence coding for the NH_2 terminal 40 amino acids of the rolA protein. GUS indicates the *uidA* coding region. The constructs were cloned in both orientation, as EcoRI fragments, in the vector pG, a derivative of pMB393 (Gage, D.J., Bobo, T. and Long, S.R. (1996) J. Bacteriology, 178, 7159-7166, see materials and methods).

Figure 2. Growth phase dependent expression of ß-glucuronidase activity in *Rhizobium leguminosarum*

containing the 86AGUS construct. Cells were grown in rich media (Tryptone-Yeast Extract rich media) until stationary phase (first sample on the negative side of the temporal axis), then diluted one hundred folds in TYR media. The optical density at 600 nm (open squares) and specific ß-glucuronidase activity (closed squares) are shown. The values are means of at least four independent experiments. SE means standard error. The construct Δ GUS, containing just the *uidA* (GUS) coding region, showed undetectable levels of specific ß-glucuronidase activity and consequently has not been represented in the figure.

Figure 3. Root nodules from *Vicia hirsuta* plants infected with *R.l. viciae* containing the 86AGUS construct were stained for ß-glucuronidase activity at different age. Panel A to D show one week old nodules. Panel A shows only few layers of cells infected, and consequently (blue)stained, just behind the meristem (indicated with an asterisk in panel E). Panel B shows that in this developmental stage the infection zone is enlarging and will occupy later (Panel C) the whole nodule. Panel D shows three nodules at three different stages of infection on the same root. Panel E shows a two weeks old nodule; note the cylindrical shape of the nodule where all the inner tissue is invaded by bacteroids expressing GUS activity. Panel F shows a three weeks old nitrogen fixing nodule where Zone II and III are (blue) stained while the senescent Zone IV is mostly not stained. Panel G shows a four weeks old nodule displaying an intense staining in the late senescent Zone closest to the root. Panel H shows a double staining for GUS activity and for starch deposition (see Materials and Methods); Note the interzone II-III indicated by the arrows. Above the interzone II-III is visible an invasion Zone II reduced in size and yet still (blue) stained; the double arrow shows (blue) spots of GUS activity closest to the root.

Figure 4. Panels A to E1: roots infected with wild type *R.l. viciae* containing either the 86AGUS construct (in panels C, D and on the right side of panels A and G) or the promintron-*iaaM-tms2* construct (panels B, E, E1, F and on the left side of panels A and G). Panels F and G are microscopic bright field images. Note that the root on the left side of panel A has been infected with bacteria containing the promintron-*iaaM-tms2* construct and has bigger nodules, clustered close to the seed and followed by a long region of the root without any nodule. The most distal part of the root has again nodules. The root on the right side of panel A has been infected with *R.l. viciae* containing the 86AGUS construct: it shows a regular emersion of nodules all along the root (amplified in panel D). Panels B and C allow to compare the size of nodules elicited with wild type (panel C) and genetically modified *Rhizobia* containing the promintron-*iaaM-tms2* construct (panel B). Panel E and E1 show the morphology of two typical nodules elicited by bacteria harbouring the promintron-*iaaM-tms2* construct: bilobate in panel E or highly clustered in panel E1. In panel F is shown a dark field microscopic view of a clarified (see Materials and Methods) bilobate nodule derived from inoculation with *R.l. viciae* containing the promintron-*iaaM-tms2* construct. In panel G the same couple of nodules shown in panel F is compared to a nodule of an identical age derived from a *R.l. viciae* harbouring the 86AGUS construct and stained for ß-glucuronidase activity.

Figure 5. *Vicia hirsuta* plants were grown in seed-growth pouches (Mega International, Minneapolis), five seeds per pouch. On the left part of panels A and B, and in panel C are shown 35 days old plants infected with *R.l. viciae* harbouring the promintron-*iaaM-tms2* construct; on the right part of panels A and B, and in panel D are shown plants infected with *R.l. viciae* containing the 86AGUS construct. Note in panels A and B

the bigger size of plants infected with the promintron-*iaaM-tms2* construct in comparison to that one of plants infected with *Rhizobia* harbouring the 86AGUS construct. Plants nodulated by *R.l. viciae* containing the 86AGUS construct have (panel D) smaller leaves, less branches and more senescent leaves per plant. Leaves with a more dark green colour are displayed by plants nodulated by *Rhizobia* harbouring the promintron-*iaaM-tms2* construct (panel C), furthermore the plants do have a more healthy appearance (compare panel C with panel D).

Figure 6. Acetylene reduction assays (ARA). Data compare ARA of 18, 27 and 31 days old plants nodulated either with *R.l. viciae* harbouring the 86AGUS construct (control) or with *Rhizobia* harbouring the promintron-*iaaM-tms2* construct. In each case the control is considered as 100% and data indicate the percentage of increase of ARA obtained from roots nodulated with *Rhizobia* containing the promintron-*iaaM-tms2* construct. Each column represents the medium value obtained from fifty plants. ARA has been expressed in arbitrary units.

Figure 7. Plant dry weight. The stem of nodulated plants was cut just above the seed and dried in an oven at 85°C overnight. Panel A shows the plant dry weight of 18, 27 and 31 days old plants nodulated with *R.l. viciae* harbouring the 86AGUS construct (control) and those infected with *Rhizobia* containing the promintron-*iaaM-tms2* construct. In each case the control is considered as 100% and indicated as zero on the abscissa axe; data indicate the percentage variation, from the corresponding control, of dry weight obtained from stems of plants nodulated with the promintron-*iaaM-tms2* construct. Each column represents the value from 50 plants.

Figure 8. Nucleotide sequence of the *rolA* promintron from Ri plasmid A4 of *Agrobacterium rhizogenes*. Sequences showing homology to: i) the -10 and -35 regions of sigma70-dependent promoters, ii) -10

region of sigma38-dependent promoters, and iii) so-called GEAR-BOX sequence are printed in bold. The arrow indicates the main initiation of transcription.

Figure 9. 29 (panels 1 and 5), 36 (panels 2 and 6), 43 (panels 3 and 7), and 50 (panels 4 and 8) day old root nodules generated on *Vicia hirsuta* plantlets by the wild type strain *R.l. viciae* RPR1105 (panels 1-4) or by the promintron-*iaaMtms2* construct (panels 5-8). Note the increase in nodule size and the different shape of nodules in 5, 6, 7 and 8 compared to control nodules in panels 1, 2, 3 and 4.

Figure 10. Root nodules of *Vicia hirsuta* plants, nodulated by the w.t. strain of R.I. viciae RPR1105 or by its derivative containing the prominitron-iaaM-tms2 construct were cut from roots at 14, 21, 28, 35, 42 and 49 day after infection. Panel B shows a diagram of the average weight per plant (Mnod(mg)/pp); standard error is indicated. Each point represents the average root nodule weight per plant and it results from at least 200 screened nodules. Panel A shows the pendence of obtained lines.

Figure 11. Thin sections (toluidine blue stained) of five week old root nodules generated by the wild type strain *R.l. viciae* RPR1105 (panels 1 and 2), by the promintron-*iaaMtms2* construct (panels 3 and 4) or by the 86AGUS construct (panel 5). Panels 2 and 4 are magnification of the nodule meristem of the two nodules shown in 1 and 3 respectively. The meristematic activity is absent in panel 5, is present at the tip of the nodule in panel 1 and 2, and is extremely active and enlarged on both sides in panel 3 and 4, where small dividing cells are present on the cap of the nodule with a large zone of recently bacterially infected cells (light stained).

Materials and Methods used are herein depicted:

Construction of recombinant plasmids

Standard techniques were used for the construction of recombinant DNA plasmids. The 86AGUS, 85-17GUS and ΔGUS constructs were subcloned in both orientations as EcoRI fragments in the plasmid vector pG, a derivative of pMB393 (Gage, D.J., Bobo, T. and Long, S.R. (1996), ibidem) obtained by deletion of a 750 bp long EcoRI fragment, and then introduced by electroporation into *Rhizobium leguminosarum* biovar *viciae*, strain LPR1105, a rifampicin resistant derivative of RCR1001 (Hooykas P.J.J. *et al.* (1977) J. Gen. Microbiol., 98, 477-484). The construct pG-promintron-*iaaM-tms2*, comprising the *rolA* promintron driving the *iaaM* and *tms2* genes is described in Example 3.

Growth conditions and GUS assay

Rhizobium l. b. *viciae* cells harbouring the different constructs were grown in TYR medium at 30°C. Growth curves were determined by measuring OD_{600}.

Bacteria, collected by centrifugation, were resuspended in 50mM Na-phosphate buffer (pH 7.0), 10mM ß-mercaptoethanol, 10mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, sonicated for 5 min twice and centrifuged at 16.000 x g for 10 min at 4°C. GUS activity was assayed by using the supernatant. Fluorimetric GUS assays were performed as described by Jefferson (Jefferson, R. (1987) Plant Mol. Biol. Report, 5, 387-405). Protein concentration in bacterial extracts was determined using the Bradford reagent (BIORAD) according to the manufacturer's instructions.

Rhizobia harbouring the different constructs were plated and selected on TYR rich media supplemented with 100 µg/ml spectinomicin, and replicated on plates containing 100 µg/ml rifampicin. Strains to be inoculated on plants were grown until stationary phase, centrifuged 5' at 12.000g, washed in PBS buffer pH 7.4 and resuspended in the same buffer. Optical Density (OD) was

measured at 600 nm and 10^5 cells were applied on each seedling and incubate for 1 hour. *Vicia hirsuta* seeds were surface sterilised in 5% hydrogen peroxide for 30' and then extensively washed with sterile bidistilled water. Seeds were allowed to swollen over-night at 4°C and then germinated on 1.5% agar plates for three days in the dark. After infection, germinated plantlets were grown in seed-germination pouches (MEGA International Minneapolis, USA) containing 10 ml of Jensen media (Vincent, J.M. (1970) I.B.P. Handbook No. 15, Oxford, Blackwell Scientific publications). Plants were grown in 70% humidity, 16/8 light/dark period at 17 or 22°C.

In situ GUS staining

Roots from nodulated plants were cut and incubated at 37°C overnight in X-Gluc Reagent mix (1mM X-Glucuronide, 0.1mM $NaPO_4$, 10mM EDTA pH7.0, 0.5mM K ferrocyanide pH 7.0, 0.5mM K ferrocyanide pH 7.0, 0.1% Triton X-100) then fixed in 4% paraformaldehyde in PBS buffer (GUS Protocols by Gallagher S.G., 1992, Academic Press, Inc.). Roots were then dehydrated in increasing concentration of absolute ethanol and clarified by immersion in a mixture 2:1 benzyl benzoate-benzyl alcohol (Sigma). For starch staining whole nodules were immersed for 20 seconds in an aqueous solution of 0.1M potassium iodide. Whole nodules were photographed with a Nikon microscope in bright-field and epipolarization optics.

Acetylene reduction assays (ARA) were performed by cutting nodulated roots (4 roots per 15 ml tube) and injecting 1 ml of acetylene for at least one hour at room temperature. Acetylene and ethylene peaks were obtained by injecting one millilitre from each tube into a Perkin Elmer Sigma 3B gas chromatograph equipped with a column Porapack Q equipped with a Perkin Elmer 561 recorder.

Example 1: The spliceosomal intron of the *rolA* gene from *Agrobacterium rhizogenes* is a promoter active in *Rhizobia.*

The DNA sequence, hereafter called promintron, spanning the *rolA* intron (which is 85 bp long and it goes from position -1 up to position -86, being +1 the A of the ATG initiation codon of the *rolA* gene) is able to drive prokaryotic expression in *Rhizobia*. A reporter gene construct (86AGUS; Fig.1) was built by fusing the coding region of the *uidA* gene (Jefferson, 1987, ibidem) to a fragment comprising the DNA sequence coding for the NH_2-terminal 40 amino acids of the RolA protein preceded by the promintron (i.e. 85 bp of the *rolA* promintron (SEQ ID No.1) plus the A immediately preceding the ATG initiation codon of the rolA coding region). GUS activity was detected by fluorimetric assays in extracts from *Rhizobium leguminosarum* biovar *viciae* (*R. l. viciae*) harbouring the construct 86AGUS (Fig.2). A similar pattern of expression (data not shown) has been obtained by using the 85-17GUS construct which comprises the promintron of SEQ ID No. 1 and a 17 bp linker (SEQ ID No. 2) and the *uidA* coding region (Fig. 1). The construct ΔGUS consisting of the *uidA* coding region by itself (Fig.1) gave no GUS activity in *Rhizobia* harbouring such construct (data not shown). *R. l. viciae* strain LPR 1105 harbouring either of the three constructs were grown in Trypton-Yeast Extract (rich media) up to stationary phase at an optical density of 1.8 at 600 nm, then diluted 100 times in the same media. Samples were taken in early, mid and late exponential phase of growth and then in early and late stationary phase of growth. GUS activity was detected only in protein extracts from *R. l. viciae* harbouring the 86AGUS (fig.2) or the 85-17GUS (data not shown) constructs while ΔGUS showed undetectable levels of β-glucuronidase activity, as untransformed *Rhizobia*. The data obtained show that, in *Rhizobia* harbouring the 86AGUS (or 85-17GUS constructs), β-glucuronidase activity declines during the exponential phase of growth and it increases in early stationary phase and it reaches the

highest value at late stationary phase of growth (Fig.2). The same experiment was repeated diluting 100 times the stationary colture grown in rich media into a minimal defined media containing a concentration of mannitol, used as carbon source, limiting for growth. After the dilution, a long period of growth lag was observed followed by an exponential phase of growth and by a stationary phase of growth reached at an optical density of 0.6. This reduced O.D. is caused by the carbon limiting condition applied. Promoters of enteric bacteria regulated by the stationary sigma factor sigma S are specifically activated under these conditions. The promintron sequence harbours a sigma S-like consensus sequence (Fig.8). The GUS activity in *Rhizobia* harbouring the 86AGUS construct was enhanced during the stationary phase of growth and the values reached were 2-times higher than those measured under stationary phase of growth reached in rich media as a consequence of the carbon starvation. Thus, gene expression driven by the promintron sequence is inversely correlated to growth rate and it is induced at the onset of the stationary phase of growth. An identical pattern of expression has been found utilising the 85-17GUS construct where the promintron sequence is separated from the *uidA* gene by a 17 base pairs polylinker shown in SEQ ID No. 2. Thus, the 85 bp long promintron has a promoter function in *Rhizobia*. Open squares represent the O.D. at 600 nm; filled squares represent GUS activity.

Example 2: <u>The promintron drives gene expression within nodules with a novel and unexpected pattern of expression.</u>

Rhizobium leguminosarum biovar *viciae* strain LPR 1105 harbouring the 86AGUS construct or the 85-17GUS or the ΔGUS, where used to infect *Vicia hirsuta* plants. The plantlets were grown under greenhouse conditions up to 60 days after planting, and at different times roots were

cut, fixed under buffered paraformaldehyde and stained for in situ GUS activity (Jefferson, 1987, ibidem), after over-night incubation in the appropriate buffer, roots were dehydrated by immerging into increasing ethanol solutions followed by toluene clarification and observed under an optical bright field microscopy. DNA fragments comprising the promintron DNA sequence shown in SEQ ID No. 1 promotes expression in the nodules within symbiotic *Rhizobia* with a characteristic and novel pattern of expression resulting in a constitutive expression of the gene of interest. For constitutive expression it is meant that expression, although timed in a specific pattern, overall it takes place in all the zones of the nodule where plant cells do contain bacteroids. This expression pattern is strikingly different from that one shown by either sigma 54 or sigma 70-like regulated promoters, for comparison see Sharma S.B. and E.R. Signer (1990) *Gene Dev.*, 4, 344-356.

Rhizobium bacteria when entering into the host plant are called bacteroids. Bacteroids are known to undergo 5 differentiation forms progressively older from stage 1 to 5. The different stages are described in Vasse, J., et al. (1990) J. Bacteriol., 172, pp. 4295-4306. In an indeterminate nodule such that of *Vicia hirsuta* the youngest bacteroids are located in the early infection Zone II, close to the tip meristem (which represents Zone I) outgrowing from the root, where bacteria are recently released from the infection threads crossing first the root hairs and then the cortical layers to reach finally the growing nodule. Much older bacteroids are located in the late infection Zone II, then in the nitrogen fixing Zone III and finally in the senescent Zone IV only ghost membrane of bacteroids are present. Expression driven by the promintron was observed in Zones II, III and IV of the nodule at any age including in the very old senescent zone in the region

most proximal to the root (see Figure 3). Thus, all plant cells harbouring bacteroids at any stage do have ß-glucuronidase activity within their bacteroids. The pictures are taken from nodules infected by *Rhizobia* containing the 86AGUS construct. An identical pattern of expression has been obtained utilising the 85-17GUS construct that gives only a slightly weaker signal. No staining has been observed with *Rhizobia* harbouring the construct ΔGUS. In Fig. 3, panel I, is presented a double staining for GUS (blue) activity and for starch (dark brown). In older nodules starch deposition occurs in Zones III and IV, but it is mostly used because its staining describes the interzone II-III (see arrow in Fig.3, panel I). The GUS staining is both above and below the interzone II-III.

Example 3: Construction of plasmid pG-promintron-*iaaM-tms2*

The recombinant plasmid pG-promintron-*iaaM-tms2* was obtained by ligating the EcoRI-KpnI fragment comprising SEQ ID No. 1 and SEQ ID No. 2, spanning the *rolA* promintron sequence, within the pG plasmid (see materials and methods) cut with EcoRI-KpnI. Afterwards the plasmid pG-promintron was cut with KpnI-HindIII and the *iaaM* and *tms2* coding sequences were introduced as KpnI-SphI and SphI-HindIII fragments, respectively. The KpnI-SphI fragment of 1775 bp (2+53+1671+49) spans the coding region of the iaaM gene from *Pseudomonas syringae* subsp. *savastanoi*. The iaaM sequence has been characterised by Yamada et al (1985, ibidem) and the sequence used contains 1773 bp (53+1671+49) from the DraI site located 53 bp before the ATG initiation codon till the SphI site 46 bp after the TAA stop codon. The SphI-HindIII fragment of 1452 (22+13+1403+14) bp spanning the *tms2* coding region from *Agrobacterium tumefaciens* pTi A6 (Klee, H. et al. (1984) P.N.A.S. USA 81, pp. 1728-1732) contains 1403 bp of coding region preceded by 14 bp and followed by 5

bp belonging to the untranslated regions of the *tms2* gene.

Consequently, the plasmid pG-promintron-*iaaM-tms2* obtained possesses the following structural features:

- a promintron fragment comprising the 85 bp of the *rolA* promintron (SEQ ID No. 1), plus 17 bp of linker sequence (SEQ ID No. 2) and having an Eco RI adapter (sequence GAATTC) at its 5' end;
- 6 bp as KpnI site (sequence GGTACC) plus extra 2 bases added as linker;
- 53 bp of 5' untranslated sequence of the *iaaM* gene from *Pseudomonas syringae* subsp. *savastanoi;*
- 1671 bp of coding region of the *iaaM* gene from *Pseudomonas syringae* subsp. *savastanoi;*
- 49 bp (46+3 bp of stop codon) of 3' untranslated trailer sequence of the *iaaM* gene from *Pseudomonas syringae* subsp *savastanoi* ending with the SpHI site;
- 22 bp consisting of a polilinker sequence (CTGCAGGTCGACTCTAGAGGAT, SEQ ID No. 3);
- 13 bp of untranslated leader region of the tms2 gene (CCAACTCAGAGAG, SEQ ID No. 4);
- 1403 bp spanning the coding region of the *tms2* gene from *Agrobacterium tumefaciens* pTiA6;
- 14 bp at the 3' end including an HindIII site (sequence TAAACATCAAGCTT, SEQ ID No. 5) being TAA the stop codon, AC sequence at the 3' of the *tms2* coding region after the stop codon, and the rest being HindIII linker sequence (HindIII site being AAGCTT).

Example 4: <u>The promintron-*iaaM-tms2* construct alters root nodule number, location and size.</u>

Vicia hirsuta plantlets were harvested at 18 days after infection with either the 86AGUS construct or the promintron-*iaaM-tms2 construct.* In the case of plants infected with *Rhizobia* harbouring the 86AGUS construct, an average number of 22 nodules per plant was observed while this number decreased at 7.5 nodules per plant in

the case of plants infected with *Rhizobia* harbouring the promintron-*iaaM-tms2* construct. Considering the control 86AGUS infected plants as 100%, the presence of the bicistronic operon in *Rhizobia* reduces the nodule number by 65%. In contrast to this feature, the nodule size is strongly increased in plants infected with *Rhizobia* harbouring the promintron-*iaaM-tms2* construct. In Figure 4, panels B and C, the size of the nodules can be compared. Not only *Rhizobia* containing the promintron-*iaaM-tms2* construct induced nodules which were bigger in size, but sometimes the nodules were also bilobate (panel E). In addition, the root nodules elicited by *Rhizobia* harbouring the promintron-*iaaM-tms2* construct, are localised mostly on the primary root (Fig.4, panel A left) and rarely on secondary roots as is the case with plants inoculated with *Rhizobia* harbouring either the 86AGUS construct or not harbouring any construct at all (Fig.4, panel A, right). Finally, Fig. 4 (panel A, left) shows that nodules elicited by *Rhizobia* harbouring the promintron-*iaaM-tms2* construct are concentrated in the root region close to the seed, followed by a large zone of the root where no nodule did occur. This was not the pattern of nodulation elicited by wild type *Rhizobium*, which consisted of small nodules distributed uniformly all along the root (Fig.4, panel A right, and panel D).

Example 5: <u>The root nodules induced by *Rhizobia* harbouring the promintron-*iaaM-tms2* construct shows an increased acetylene reduction activity (ARA)</u>

Nodulated roots infected with wild type *Rhizobium leguminosarum* biovar *viciae* strain LPR1105 or the same strain harbouring either the 86AGUS or the promintron-*iaaM-tms2* construct were followed for one month after infection and assayed for the ability to reduce acetylene to ethylene. This is the most commonly used indirect enzymatic assay to evaluate the ability of the nitrogenase complex to reduce atmospheric nitrogen to

ammonia. Eight independent experiments using up to fifty plants per point have been carried out at two different temperatures (17°C and 22°C). Results show that the promintron-*iaaM-tms2* construct induces the formation (at 22°C) of root nodules able to reduce acetylene to ethylene more efficiently than the control root nodules generated either by the wild type *Rhizobia* or by *Rhizobia* harbouring either the 86AGUS or the 85-17GUS. Thus, the presence of the promintron-*iaaM-tms2* construct increases the capacity of nitrogen fixation of root nodules. Samples were taken at 18, 27 and 31 days after inoculation of germinated seeds with bacteria. Acetylene reduction activity (ARA) was followed at the Gas Cromatograph (see Materials and Methods) and expressed in arbitrary units. In Fig. 6 the media of the value of the control (at least 50 plants in each case) are taken as 100%. Each column indicate the percentage of increase compared to the controls of ARA from roots nodulated with the promintron-*iaaM-tms2* construct. In function of time, the percentage of ARA increases from 30%, at 18 days, up to 240% more than controls at 31 days.

Example 6: Plant growth and biomass

As shown in Fig. 5, 35 days old *Vicia hirsuta* plants nodulated by *Rhizobia* containing the promintron-*iaaM-tms2* construct are bigger in size (more than 20% increase). Furthermore, these plants are not only bigger, but also altered in their phenotype having dark green leaves and a better vigour in comparison to sister plants inoculated with the wild type *R.l. viciae*. In Figure 7 are listed the dry weights of plants 18, 27 and 31 days old. The dry weight of control plants is considered 100% at any age and indicated as zero. It could be observed a significant decrease (i.e. 24%) in the average dry weight of plants nodulated with *Rhizobia* containing the promintron-*iaaM-tms2* construct at 18 days, when the influence on plant growth of seed storage proteins is

still important. In contrast, 27 and 31 days old plants, when the nitrogen contained in the seeds is mostly exhausted, shows a 35% and 44%, respectively, increase in dry weight of plants nodulated with *Rhizobia* harbouring the promintron-*iaaM-tms2* construct in comparison to the dry weight of plants nodulated by the wild type strain of *Rhizobia* or of *R.l. viciae* containing the 86AGUs or the 85-17GUS constructs.

Example 7: Measurements of acetylene reduction

Acetylene reduction assays were performed to compare root nodules generated on *Vicia hirsuta* by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105 or from its derivative containing the *promintron-iaaMtms2* construct. Up to 23 days after infection the two strains reduce the same amount of acetylene at a similar rate. Five weeks after infection the meristematic activity is arrested in control nodules while is still generating newly infected cells after 8 weeks in nodules inoculated with bacteria containing the *promintron-iaaMtms2* construct. At 39 days after infection the acetylene reduction reach a 100% increase when the *promintron-iaaMtms2* construct is compared to the wild type strain.

Example 8: Increase of stem dry weight

An analysis of *Vicia hirsuta* stem dry weight was performed to compare *Vicia hirsuta* plants nodulated by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105 with its derivative containing the *promintron-iaaMtms2* construct. The plant biomass was substantially unaffected up to the 23 day after infection. However a 15% increase at 51 days after infection was observed when the *promintron-iaaMtms2* construct is compared to the wild type strain.

Example 9: Total nitrogen content analysis

The analysis of *Vicia hirsuta* stem dry weight was followed by mineralized total nitrogen content analysis.

The total nitrogen content, as elementary N, was measured according to the method of Dumas (Simonne, et al., J. Sci. Food Agric. 73, 39-45) using a Nitrogen Analyzer Macro-N (Foss Heraeus Analysensysteme, Hanau Germany). The total nitrogen concentration is unaffected up to 51 days after infection on nodulated *Vicia hirsuta* plants both with the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105, and with its derivative containing the *promintron-iaaMtms2* construct.

Example 10: Analysis of the root nodule weight

An analysis of the root nodule weight was performed to compare plants nodulated by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105, with its derivative containing the *promintron-iaaMtms2* construct. Figure 9 shows 29, 36, 43 and 50 day old root nodules (panels 1,5; 2,6; 3,7; 4,8; respectively) generated on *Vicia hirsuta* plantlets by the wild type strain *R.l. viciae* 1004 (panels 1-4) or by the promintron-*iaaMtms2* construct (panels 5-8). The total mass of nodules is unaffected up to four week after infection with both strains. It reaches a two fold increase 42 days after infection when the *promintron-iaaMtms2* construct is compared to the wild type strain. Figure 10B shows a diagram to compare data of nodule weight up to day 49 after infection among the two strains. The panel of Figure 10A shows that the slope of the interpolating line is 0.26 for the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105, whereas a slope value of 0.46 is obtained when the *promintron-iaaMtms2* construct was used.

Example 11: Analysis of meristematic activity

An analysis of thin sections stained with 0,02% toluidine blue of five week old root nodules was performed to compare plants nodulated by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105 with its derivative containing the *promintron-iaaM-tms2*

construct or the 86AGUS construct. Figure 11 shows that the meristematic activity and newly infected cells are absent in nodules generated by the 86AGUS containing bacteria. However they are present but restricted to the very tip of the root when the wild type strain RPR1105 was used; whilst meristematic activity is still very active with many dividing (small) cells present at the cap of the nodule, with many new infected (light stained) cells when the *promintron-iaaMtms2* construct was used. Therefore it is evident that, at the same age, both the size and the shape of generated nodules in the three samples are statistically different.

Example 12: Measurements of acetylene reduction in French bean

Acetylene reduction assays were performed to compare French bean plants nodulated by the wild type strain CE3 of *Rhizobuim etli* or by its derivative containing the *promintron-iaaM-tms2* construct. In contrast to the *Vicia hirsuta* indeterminate type of nodule, French bean produces a determinate type of nodule, namely a nodule missing a persistent meristem, similar to those generated on soybean roots. At 23 days after infection the acetylene reduction rates are identical irrespective to the strain utilised for root infection. At 43 days after infection French bean plants inoculated with bacteria containing the *promintron-iaaMtms2* construct show a 100% increase when compared to the wild type *R. etli* CE3.

Example 13: Increase of stem dry weight in French bean

French bean stem dry weight analysis was performed to compare plants nodulated by the wild type strain CE3 of *Rhizobuim etli* or by its derivative containing the *promintron-iaaMtms2* construct. The stem dry weight is identical up to day 23 after the infection irrespective to the strain utilised, while at day 48 the stem dry

weight of plants nodulated by the CE3 strain containing the *promintron-iaaMtms2* construct is increased by 15% when compared to 48 days old French bean plants infected with the wild type strain of *R. etli* CE3.

Example 14: Analysis of the root nodule weight in French bean

French bean nodule weight analysis was performed to compare plants nodulated by the wild type strain CE3 of *Rhizobuim etli* or by its derivative containing the *promintron-iaaMtms2* construct. The nodule weight is identical up to day 23 after the infection, irrespective to the strain utilised, while at day 48 the nodule mass of plants nodulated by the CE3 strain containing the *promintron-iaaMtms2* construct is increased by 50% when compared to 48 days old French bean plants infected with the wild type strain of *R. etli* CE3.

CLAIMS

1. Use of the promintron sequence of the *rolA* gene from *Agrobacterium rhizogenes* as in SEQ ID NO. 1, or of DNA sequences comprising said promintron sequence, or of functional homologous or portion thereof, to induce the expression of a DNA coding sequence, in recombinant bacteria during exponential, post-exponential and stationary phase of growth, and in bacteroids within root nodules, said coding DNA sequence being under the control of said promintron sequence.

2. Use of the promintron sequence according to claim 1 wherein said recombinant bacteria belong to either the *Enterobacteriaceae* or the *Rhizobiaceae* families.

3. Use of the promintron sequence according to claim 2 wherein said recombinant bacteria belonging to either the *Enterobacteriaceae* or the *Rhizobiaceae* families are *E. coli*, *Rhizobia* or *Agrobacteria*.

4. Use of the promintron sequence according to claim 3 wherein said recombinant bacteria are of the *Rhizobia* genus, either within symbiotic root nodules or in a free living status.

5. Use of the promintron sequence according to claim 4 wherein said recombinant bacteria of the *Rhizobia* genus within symbiotic root nodule, are either bacteroids of stage I, II, III, IV, V, or *Rhizobia* present in the apoplastic space, or *Rhizobia* present in the senescence zone, or *Rhizobia* present in the nitrogen fixing zone, or *Rhizobia* present in the invasion zone.

6. A recombinant DNA molecule comprising the promintron sequence according to claim 1, or functional homologous or portion thereof, and covalently linked to the 3′ end of said promintron sequence, a DNA coding sequence, said recombinant DNA molecule being

either harboured by episomal elements or integrated in the bacterial genome.

7. The recombinant DNA molecule according to claim 6 wherein said DNA coding sequence is either a monocistronic or a polycistronic transcriptional unit.

8. The recombinant DNA molecule according to claim 6 or 7 wherein said DNA coding sequence encodes a protein involved in plant hormone synthesis and/or metabolism.

9. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes a protein involved in plant hormone auxin synthesis and/or metabolism.

10. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes a protein involved in the synthesis and/or metabolism of the auxin IAA or of the auxin indolethanol.

11. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes the iaaM protein from *P. syringae* subsp. *savastanoi* or an homologous thereof.

12. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes the *tms2* protein from *A. tumefaciens* or an homologous thereof.

13. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes both the *iaaM* and the *tms2* coding regions of claim 11 and 12, respectively.

14. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes the indolepyruvate decarboxylase from *Enterobacter cloacae* or an homologous thereof.

15. Genetically engineered bacteria comprising the recombinant DNA molecule according to claims from 6 to 14.
16. Use of the recombinant DNA molecule according to claims from 6 to 14 to significantly increase the size of nodules of a plant.
17. Use of the recombinant DNA molecule according to claim 16 wherein said statistically significant increase of the nodule size is of at least 20%.
18. Use of the recombinant DNA molecule according to claims from 6 to 14 to significantly increase the capacity to fix nitrogen of a nodulated plant.
19. Use of the recombinant DNA molecule according to claim 18 wherein said statistically significant increase of the capacity to fix nitrogen is of at least 20%.
20. Use of the recombinant DNA molecule according to claims from 6 to 14 to significantly increase the plant biomass production.
21. Use of the recombinant DNA molecule according to claim 20 wherein said statistically significant increase of the plant biomass production is of at least 10%.
22. Legume plant infected by bacteria harbouring the recombinant DNA molecule according to claims from 6 to 14 and having a significant increase of the size of nodules, and/or of the nodule capacity to fix nitrogen, and/or of the plant biomass, and/or of the ability to fix nitrogen.

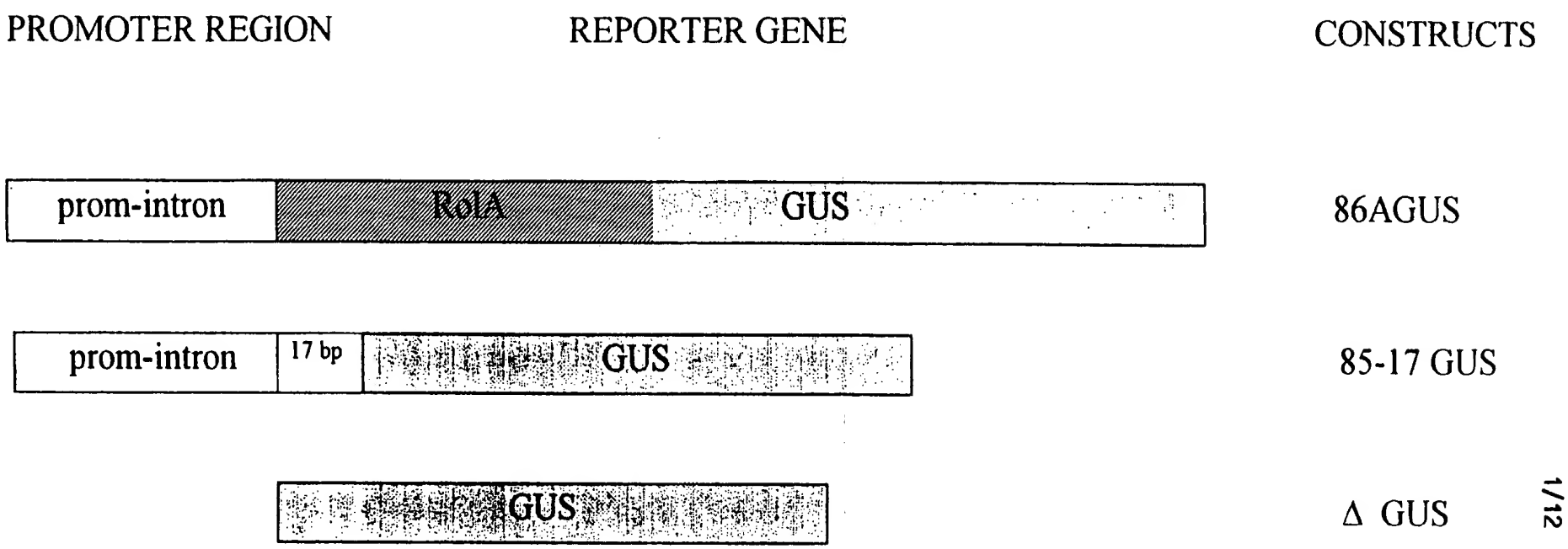

PROMOTER REGION
REPORTER GENE
CONSTRUCTS
prom-intron
RolA
GUS
86AGUS
prom-intron
17 bp
GUS
85-17 GUS
GUS
Δ GUS

FIG. 1

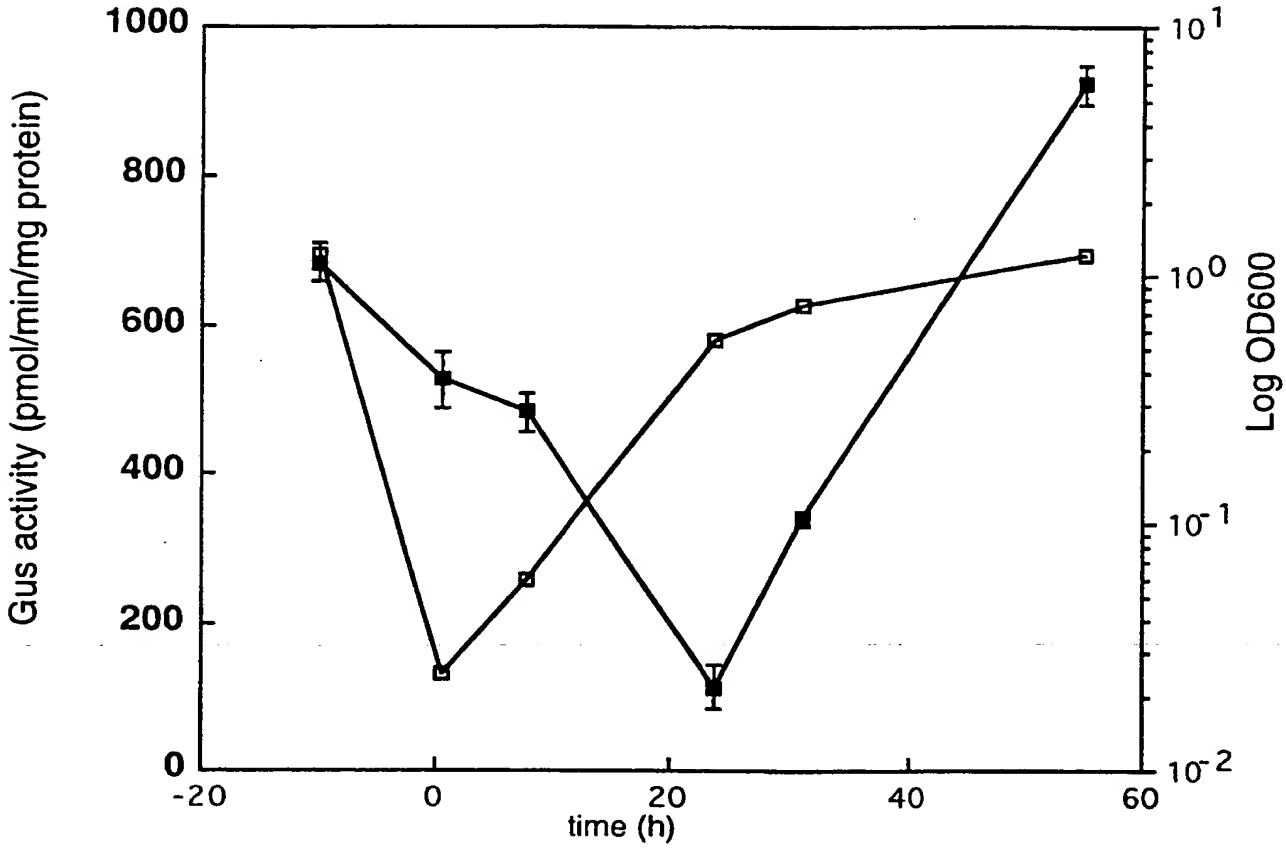

1000
800
600
400
200
0
Gus activity (pmol/min/mg protein)
-20
0
20
40
60
time (h)
10^1
10^0
10^{-1}
10^{-2}
Log OD600

FIG. 2

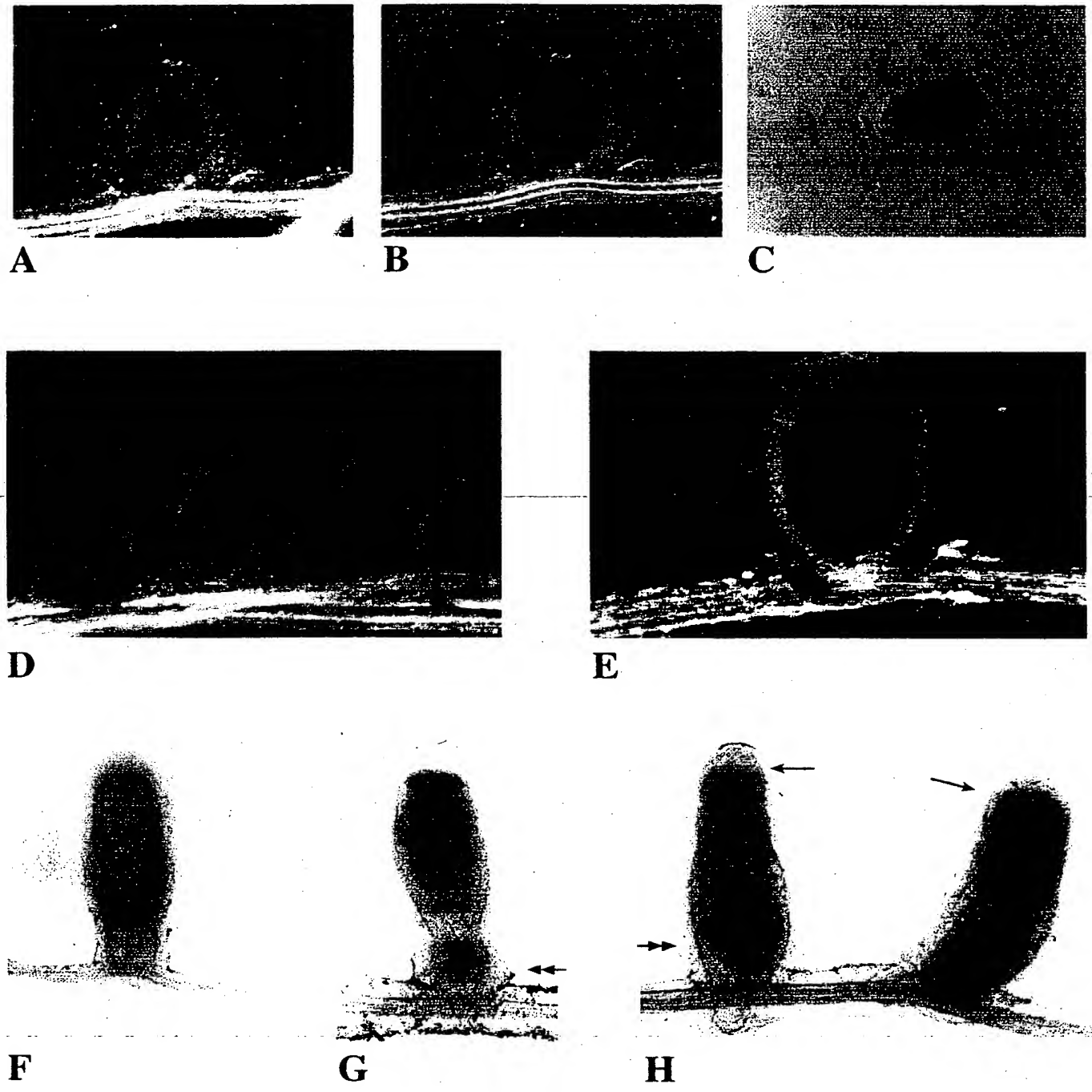
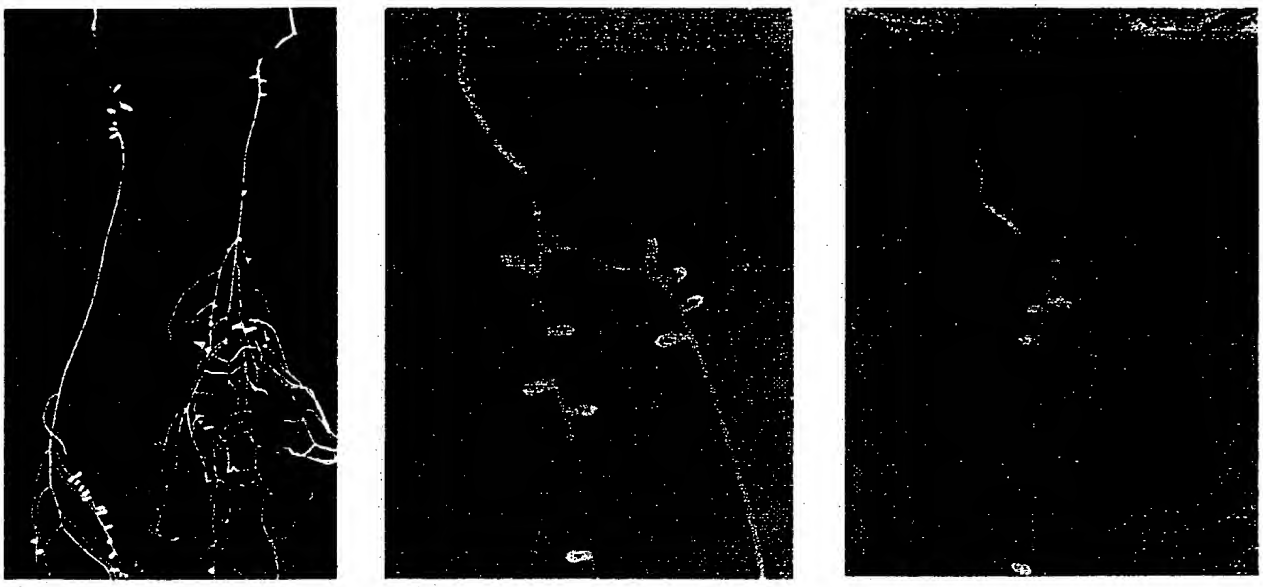
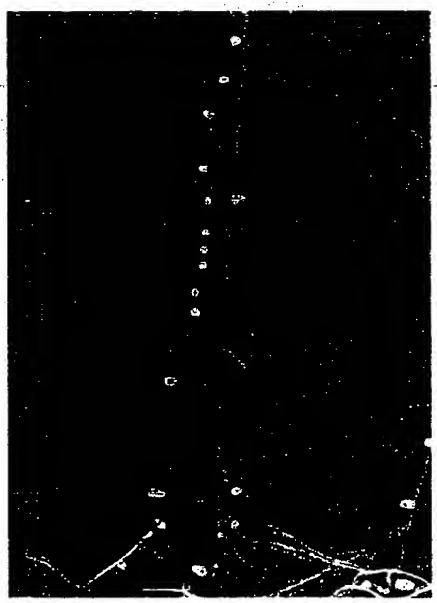


FIG. 3

FIG. 4



A B C



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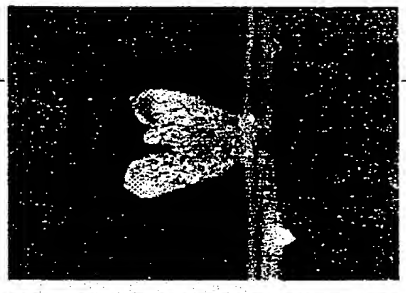
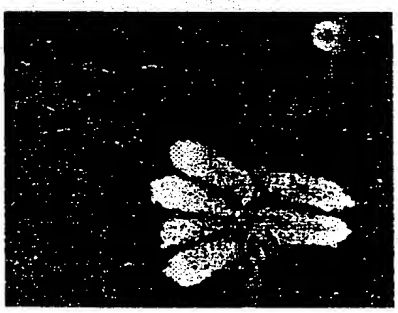


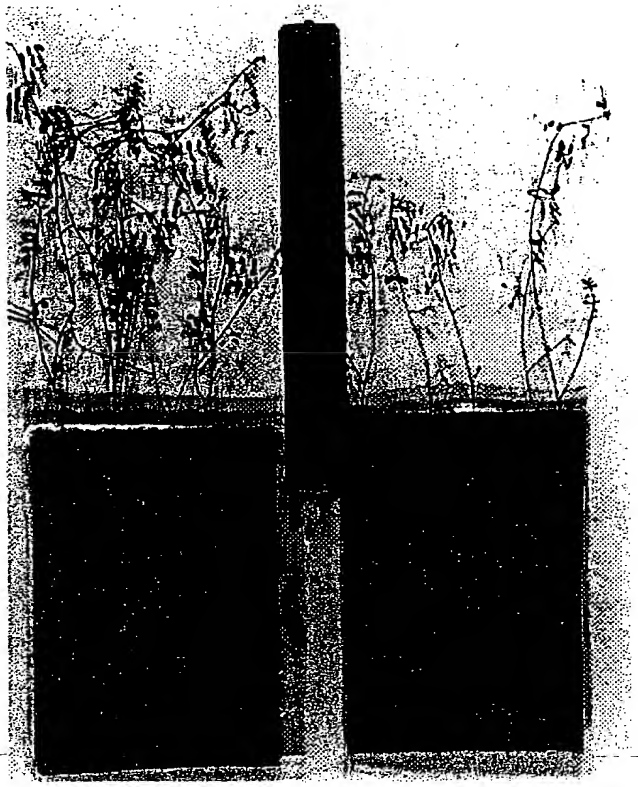
E1



E

F

G



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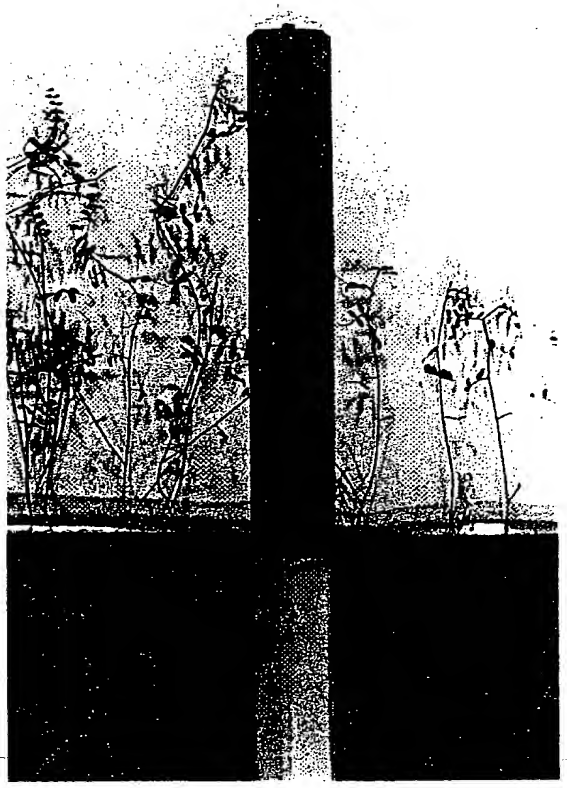
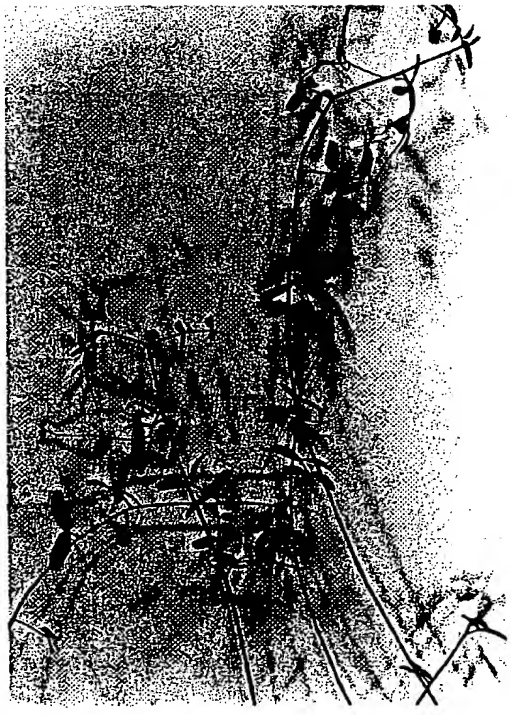


B

C



D

FIG. 5

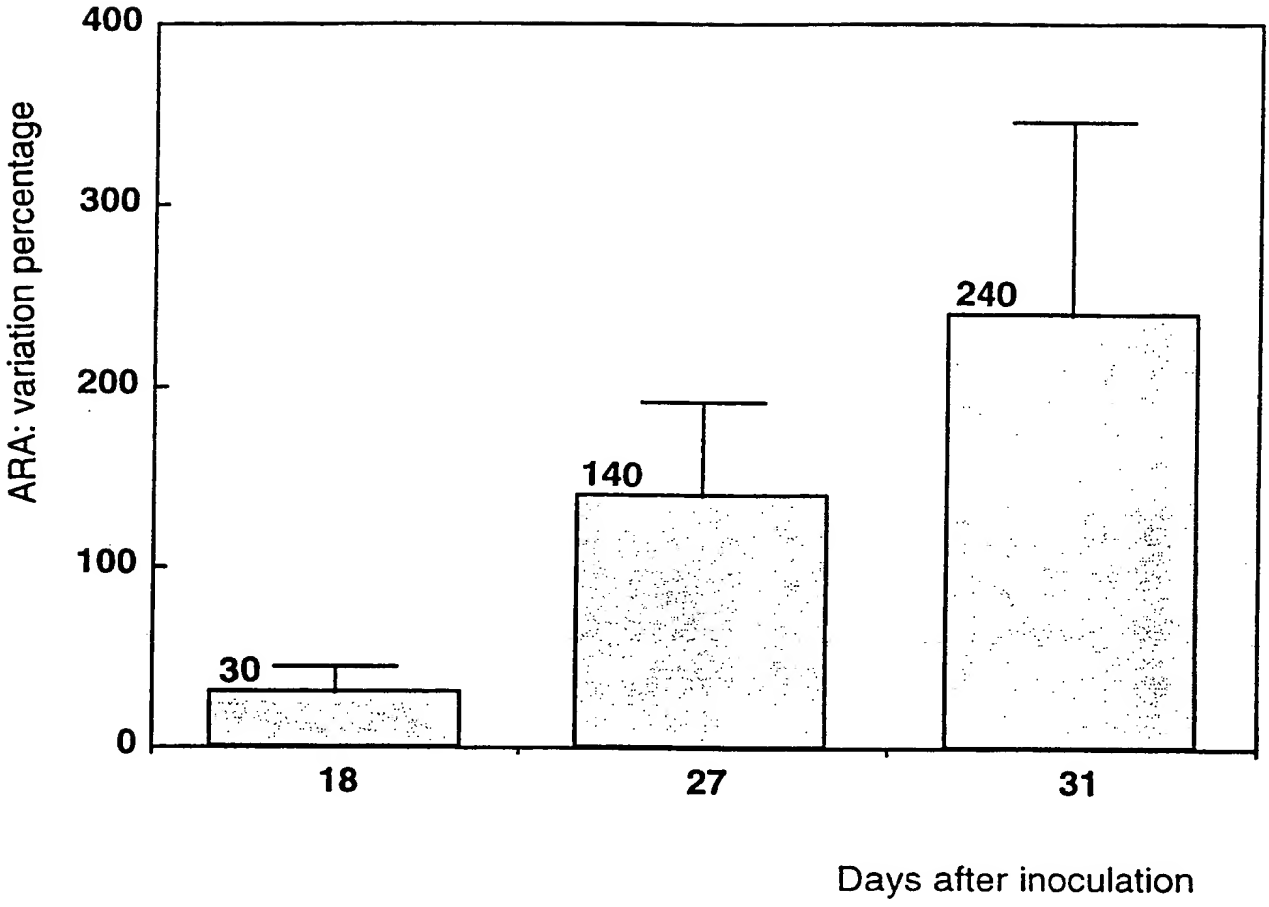

400
300
200
100
0
ARA: variation percentage
30
140
240
18
27
31
Days after inoculation

FIG. 6

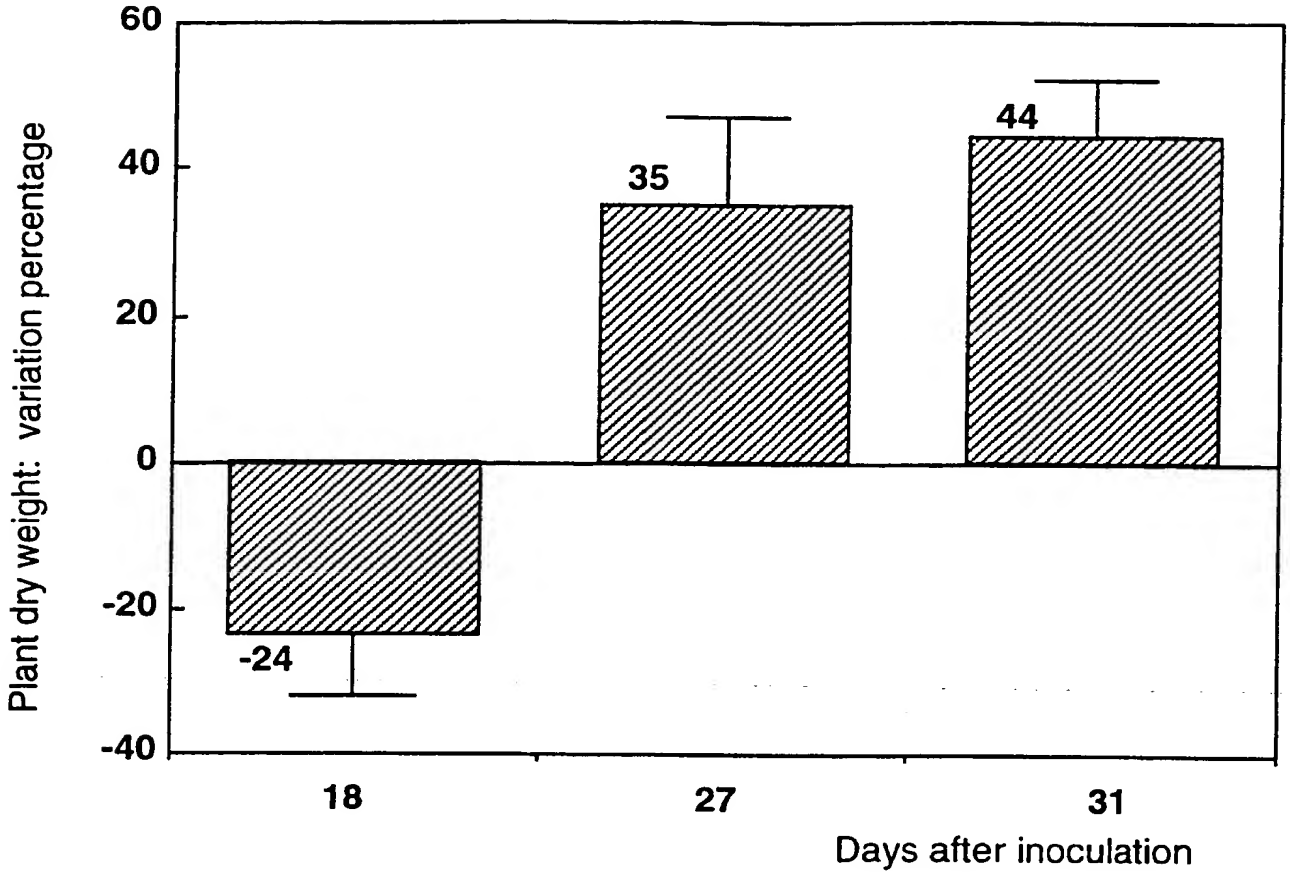

60
40
20
0
-20
-40
Plant dry weight: variation percentage
-24
35
44
18
27
31
Days after inoculation

FIG. 7

+1

GTGAGTGTGGTTGTAGGTTCAATTATTACTATTT**TTGAAG**CTGTGTATT-TCCCTTTT**TCTAATAT**GCACCTATTTCATGTTTC**AG**AATG// rolA A4 coding region

S.S. S.S.

TTGAAT_____17bp _____TATAAT
-35 -10

σ^{70}CONSENSUS SEQUENCE

CTATACT
-10

σ^{38} CONSENSUS SEQUENCE

CGGCAAGT

GEAR BOX

FIG. 8

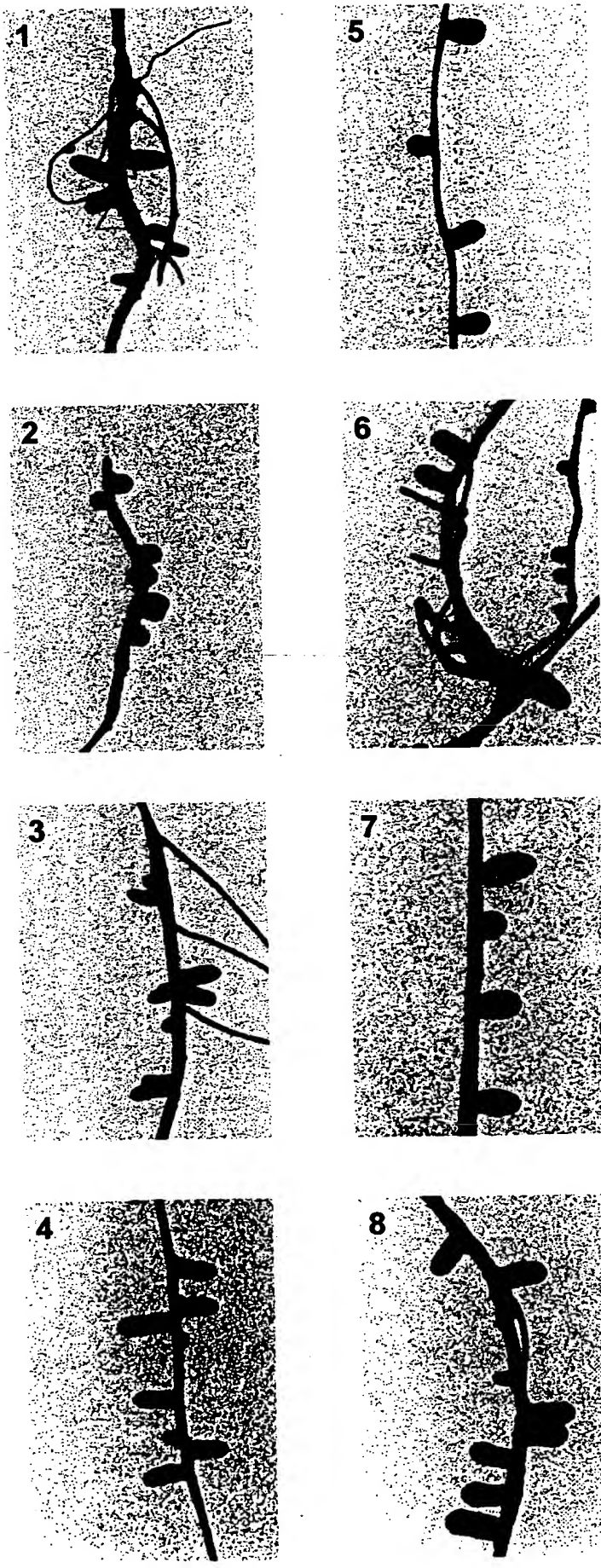


FIG. 9

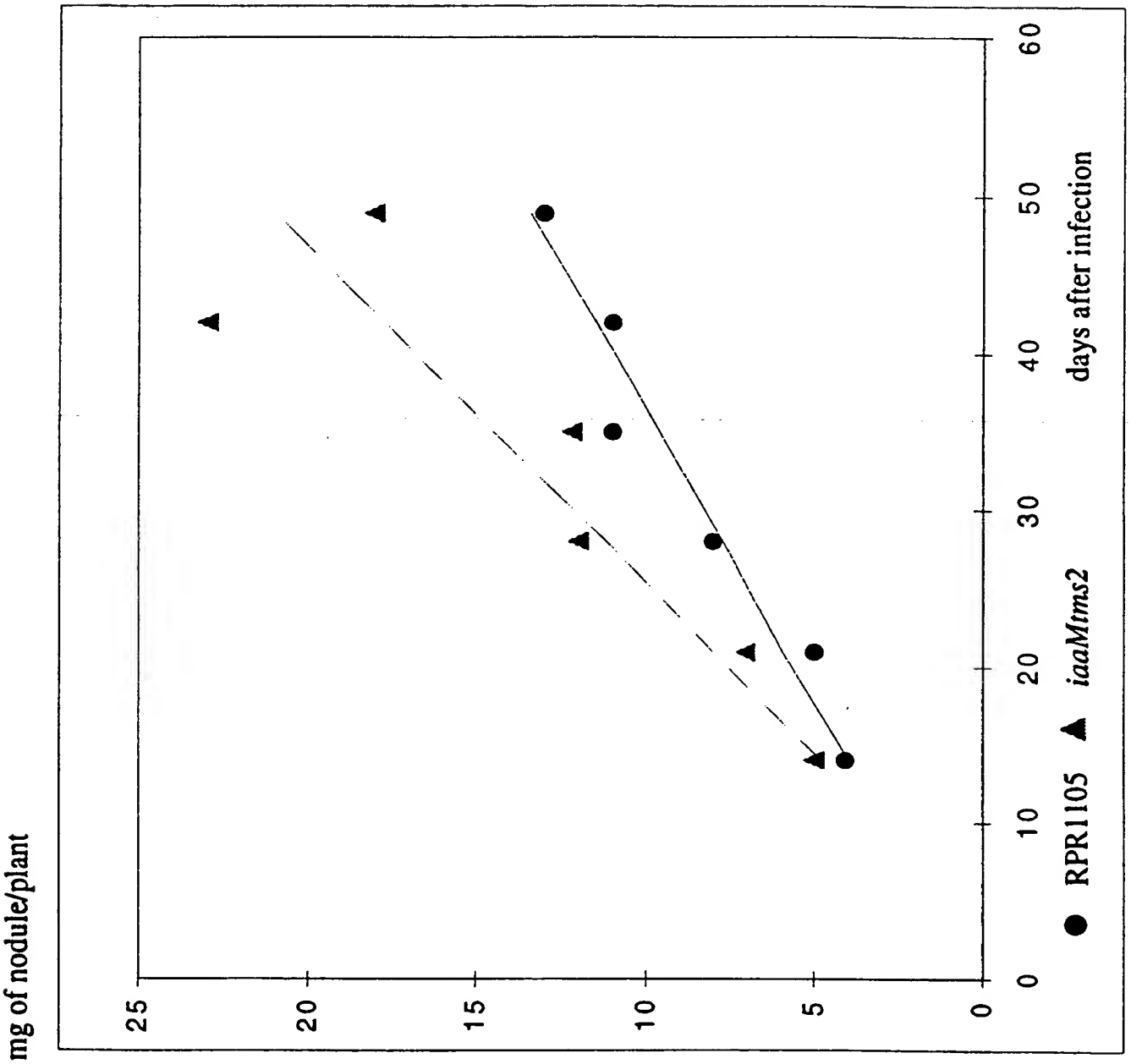
Days A. I.	Mnod(mg)/pp RPR 1105	*iaaMtms2*
14	4 ± 1	5,0 ± 0,4
21	5 ± 1	7 ± 2
28	8 ± 2	12 ± 3
35	11 ± 1	12,2 ± 0,3
42	11 ± 4	23 ± 6
49	13 ± 3	18 ± 4

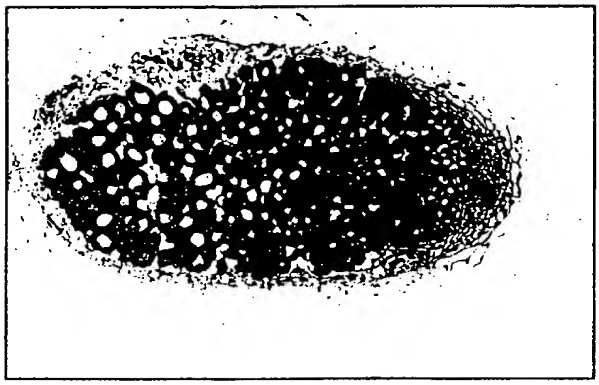
pendence	RPR 1105	0,26700	±	3,E-05
intercept	RPR 1105	0,26	±	0,03

pendence	*iaaMtms2*	0,4620	±	1,E-04
intercept	*iaaMtms2*	-1,69	±	0,1

FIG. 10A

FIG. 10B


mg of nodule/plant
25
20
15
10
5
0
0
10
20
30
40
50
60
days after infection
RPR1105
iaaMtms2



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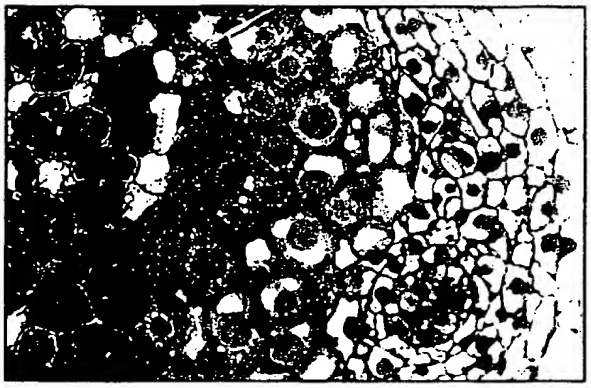

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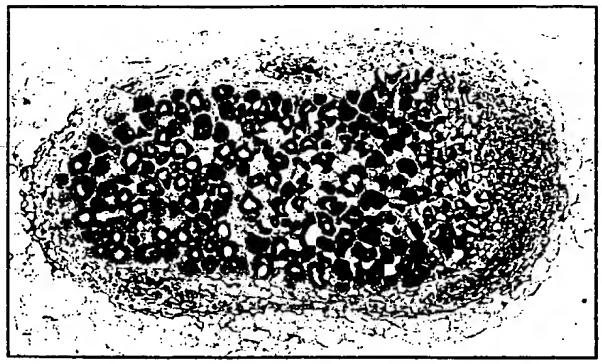

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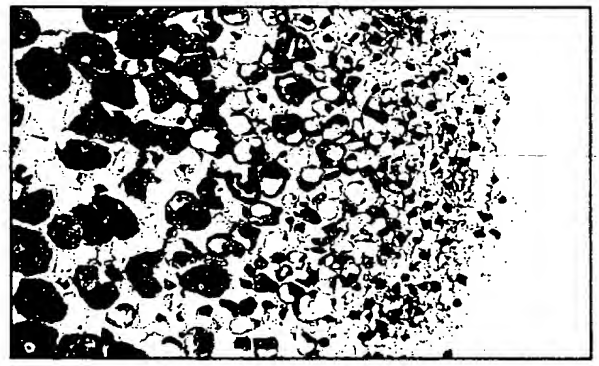
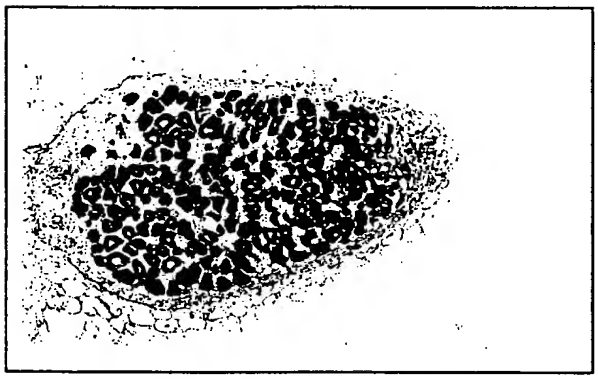


4



5

FIG. 11

SEQUENCE LISTING

<110> G.IN.E.ST.R.A. s.c.ar.l.
Consiglio Nazionale delle Ricerche
Spena, Angelo
Defez, Roberto

<120> Method to control gene expression in bacteria, namely Rhizobiaceae, to improve root nodule development, nitrogen fixation and plant biomass production

<130> PCT

<140>
<141>

<160> 5

<170> PatentIn Ver. 2.1

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<211> 85
<212> DNA
<213> Agrobacterium rhizogenes

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aatatgcacc tatttcatgt ttcag 85

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<211> 17
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: linker sequence

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<210> 5
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<220>
<223> Description of Artificial Sequence: linker sequence

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taaacatcaa gctt 14

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IT 99/00355

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/74 C12N15/31 A01N63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAGRELLI, A., ET AL.: "Splicing of the rolA transcript of agrobacterium rhizognes in Arabidopsis" SCIENCE, vol. 266, 23 December 1994 (1994-12-23), pages 1986-1988, XP002099126 cited in the application	6-8
A	the whole document	1-5,9-22

X	EP 0 204 590 A (AGRONOMIQUE INST NAT RECH) 10 December 1986 (1986-12-10)	6-8
A	* fig. 2, nts 9659 - 9743 *	1,2

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2000	29/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

Form PCT:ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SCHMÜLLING, T., ET AL.: "Promoters of the rolA, B, and C genes of Agrobacterium rhizogenes are differentially regulated in transgenic plants" THE PLANT CELL, vol. 1, July 1989 (1989-07), pages 665-670, XP002099127 page 669, right-hand column	6,7
X	DEHIO. C., ET AL.: "Stable expression of a single-copy rolA gene in transgenic Arabidopsis thaliana plants allows an exhaustive mutagenic analysis of the transgene-associated phenotype" MOL. GEN. GENET., vol. 241, 1993, pages 359-366, XP002099128 page 360, right-hand column, last paragraph; figure 5	6-8
A	US 5 085 588 A (LONG SHARON R ET AL) 4 February 1992 (1992-02-04) column 2, line 65 - line 68	1-22
A	EP 0 159 779 A (AGRIGENETICS RES ASS) 30 October 1985 (1985-10-30) the whole document	1,2
A	EP 0 130 047 A (AGRIGENETICS RES ASS) 2 January 1985 (1985-01-02) page 10, last paragraph -page 12, paragraph 1	1,2
A	KONDOROSI, A., ET AL.: "Molecular basis of legume-Rhizobium interactions: potentialsfor improving symbiotic nitrogen fixaton" GRASSLAND FOR OUR WORLD,1993, pages 381-384, XP002099129 page 383	8-22
A	COSTACURTA, A., ET AL.: "Synthesis of phytohormones by plant-associated bacteria" CRITICAL REVIEWS IN MICROBIOLOGY, vol. 21, no. 1, 1995, pages 1-18, XP002099130 the whole document	8-22

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ERNSTSEN, A., ET AL.: "Endogenous indoles and the biosynthesis and metabolism of indole-3-acetic acid in cultures of Rhizobium phaseoli" PLANTA, vol. 171, 1987, pages 422-428, XP002099131 cited in the application the whole document	8-22
A	HIRSCH, A.M., ET AL.: "Plant hormones and nodulation: what's the connection?" PLANT MOLECULAR BIOLOGY, vol. 26, 1994, page 5-9 XP002099132 cited in the application the whole document	8-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IT 99/00355

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0204590 A	10-12-1986	US 5182200 A	26-01-1993
		US 5366887 A	22-11-1994
		US 5466792 A	14-11-1995
		US 5824866 A	20-10-1998
		US 5543501 A	06-08-1996
US 5085588 A	04-02-1992	NONE	
EP 0159779 A	30-10-1985	US 4771002 A	13-09-1988
		AT 64756 T	15-07-1991
		AU 581877 B	09-03-1989
		AU 3910285 A	05-09-1985
		JP 60221080 A	05-11-1985
		JP 6315389 A	15-11-1994
		JP 7112433 B	06-12-1995
EP 0130047 A	02-01-1985	NZ 208557 A	29-11-1988
		US 5001061 A	19-03-1991
		US 5008194 A	16-04-1991
		US 5137816 A	11-08-1992
		ZA 8404684 A	27-02-1985

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* PCT24190

B x No. I TITLE OF INVENTION: METHOD TO CONTROL GENE EXPRESSION IN BACTERIA, NAMELY *RHIZOBIACEAE*, TO IMPROVE ROOT NODULE DEVELOPMENT, NITROGEN FIXATION AND PLANT BIOMASS PRODUCTION

Box No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.
The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

G.IN.E.ST.R.A. Società Consortile a r.l.
Piazza Caduti, 20
'100 VERONA - ITALY

[ ] This person is also inventor

Telephone No.
047/347164

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [X] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.
The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

CONSIGLIO NAZIONALE DELLE RICERCHE
P.le Aldo Moro 7
00185 ROMA - ITALY

This person is:

[ ] applicant only

[ ] applicant and inventor

[ ] inventor only *(If this check-box is marked, do not fill in below.)*

tate *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [X] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[X] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: [X] agent [ ] common representative

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country.)*
BANCHETTI Marina - CAPASSO Olga - de SIMONE Domenico - FIORUZZI Maria Augusta - IANNONE Carlo Luigi - TALIERCIO Antonio - ZANARDO Giovanni - ING. BARZANO' & ZANARDO ROMA S.p.A. - Via Piemonte 26 - 00187 ROMA - ITALY

Telephone No.
06/4743241

Facsimile No.
06/4870273

Teleprinter No.
625579

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

See Notes to the request form

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

DEFEZ Roberto
Istituto Internazionale di Genetica e Biofisica
Via Marconi 10
80125 NAPOLI - ITALY

This person is:
[ ] applicant only
[X] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

SPENA Angelo
Via Zamboni 38/A
37100 VERONA - ITALY

This person is:
[ ] applicant only
[X] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: ITALY

State *(that is, country)* of residence: ITALY

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [X] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:
[ ] applicant only
[ ] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

This person is:
[ ] applicant only
[ ] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

See Notes to the request form

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes: at least one must be marked):*

Regional Patent

X	AP	**ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, TZ Tanzania , ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
X	EA	**Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
X	EP	**European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
X	OA	**OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea,GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other king of protection or treatment desired, specify on dotted line)*

National Patent *(if other king of protection or treatment desired, specify on dotted line):*

X	AE	United Arab Emirates	X	LR	Liberia
X	AL	Albania	X	LS	Lesotho
X	AM	Armenia	X	LT	Lithuania
X	AT	Austria	X	LU	Luxembourg
X	AU	Australia	X	LV	Latvia
X	AZ	Azerbaijan	X	MD	Republic of Moldova
X	BA	Bosnia and Herzegovina	X	MG	Madagascar
X	BB	Barbados	X	MK	The former Yugoslav Republic of Macedonia
X	BG	Bulgaria	X	MN	Mongolia
X	BR	Brazil	X	MW	Malawi
X	BY	Belarus	X	MX	Mexico
X	CA	Canada	X	NO	Norway
X	CH and LI	Switzerland and Liechtenstein	X	NZ	New Zealand
X	CN	China	X	PL	Poland
X	CU	Cuba	X	PT	Portugal
X	CZ	Czech Republic	X	RO	Romania
X	DE	Germany	X	RU	Russian Federation
X	DK	Denmark	X	SD	Sudan
X	DM	Dominica	X	SE	Sweden
X	EE	Estonia	X	SG	Singapore
X	ES	Spain	X	SI	Slovenia
X	FI	Finland	X	SK	Slovakia
X	GB	United Kingdom	X	SL	Sierra Leone
X	GD	Grenada	X	TJ	Tajikistan
X	GE	Georgia	X	TM	Turkmenistan
X	GH	Ghana	X	TR	Turkey
X	GM	Gambia	X	TT	Trinidad and Tobago
X	HR	Croatia	X	TZ	Tanzania
X	HU	Hungary	X	UA	Ukraine
X	ID	Indonesia	X	UG	Uganda
X	IL	Israel	X	US	United States of America
X	IN	India	X	UZ	Uzbekistan
X	IS	Iceland			
X	JP	Japan	X	VN	Viet Nam
X	KE	Kenya	X	YU	Yugoslavia
X	KG	Kyrgyzstan	X	ZA	South Africa
			X	ZW	Zimbabwe
X	KP	Democratic People's Republic of Korea	Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:		
X	KR	Republic of Korea			
X	KZ	Kazakhstan			
X	LC	Saint Lucia			
X	LK	Sri Lanka			

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 09/11/98 9 NOVEMBER 1998	98830674.2		EUROPEAN PATENT OFFICE	
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

** Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(Day/month/year)*	Number	Country *(or regional Office)*
07/04/99	EP 98 83 0674	EPO

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	4
description (excluding sequence listing part)	:	31
claims	:	3
abstract	:	1
drawings	:	12
sequence listing part of description	:	2
Total number of sheets	:	53

This international application is **accompanied by** the item(s) marked below:

1. [X] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] priority document(s) identified in Box No. VI as item(s):
6. [ ] translation of international application into (language):
7. [ ] separate indications concerning deposited microorganism or other biological material
8. [X] nucleotide and/or amino acid sequence listing in computer readable form
9. [X] other *(specify)*: STATEMENT

Figure of the drawings which should accompany the abstract: 6

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

CAPASSO Olga

For receiving Office use only

1. Date of actual receipt of the purported international application	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference PCT24190

Applicant G.IN.E.ST.R.A. et al.

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE — 60.000 [T]

2. SEARCH FEE — 1.829.775 [S]

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 53 sheets.

rst 30 sheets — 799.680 [b1]

23 remaining sheets x 19.363 additional amount = 445.350 [b2]

Add amounts entered at b1 and b2 and enter total at B — 1.245.030 [B]

Designation Fees

The international application contains _____ designations.

number of designation fees x amount of designation fee payable *(maximum 10)* = 1.839.460 [D]

Add amounts entered at B and D and enter total at I — 3.084.490 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* — [P]

5. TOTAL FEES PAYABLE — 4.974.265 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

[ ] The designation fees are not paid at this time.

MODE OF PAYMENT

[ ] authorization to charge deposit account (see below)
[X] bank draft
[ ] coupons
[ ] cheque
[ ] cash
[ ] other *(specify):*
[ ] postal money order
[ ] revenue stamps

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ _____

[ ] is hereby authorized to charge the total fees indicated above to my deposit account.

[ ] is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

[ ] is hereby authorized to charge the fees for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No. | Date *(day/month/year)* | Signature

STATEMENT

The undersigned hereby declares that the information recorded in computer readable form is identical to the written sequence listing.

(CAPASSO Olga)

Rome, November 8, 1999

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

BANCHETTI, Marina
Ing. Barzano' & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Rome
ITALIE

Date of mailing (day/month/year) 26 January 2000 (26.01.00)	
Applicant's or agent's file reference PCT24190	**IMPORTANT NOTIFICATION**
International application No. PCT/IT99/00355	International filing date (day/month/year) 08 November 1999 (08.11.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 09 November 1998 (09.11.98)
Applicant G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
09 Nove 1998 (09.11.98)	98830674.2	EP	25 Janu 2000 (25.01.00)

The International Bureau of WIPO
34, ch min des Col mbettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Christine Carrié

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT

To:
BANCHETTI, Marina et al.
BARZANO & ZANARDO ROMA S.P.A.
26, Via Piemonte
00187 ROMA
ITALIE

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*: 21.06.00

Applicant's or agent's file reference: PCT24190

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IT 99/00355	08/11/1999	09/11/1998

Applicant: G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al.

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

   01/06/2000

2. This date of receipt is:

   ☒ the actual date of receipt of the demand by this Authority (Rule 61.1(b)).

   ☐ the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).

   ☐ the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. ☐ **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

   ☐ *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on: _____

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/
European Patent Office
D-80298 Munich
Tel. (+49-89) 2399-0, Tx: 523656 epmu d
Fax: (+49-89) 2399-4465

Authorized officer
LUOMA M P
Tel. (+49-89) 2399-8929

EUROPÄISCHES PATENTAMT - EUROPEAN PATENT OFFICE - OFFICE EUROPÉEN DES BREVETS

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

by fax and post

PCT

To:

BANCHETTI, Marina et al.
BARZANO & ZANARDO ROMA S.P.A.
26, Via Piemonte
00187 ROMA
ITALIE
FAX NO: 06/4870273

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	08.02.01
Applicant's or agent's file reference: PCT24190	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IT99/00355	08/11/1999	09/11/1998

Applicant
G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Büchler, S Tel.+49 89 2399-8090

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PCT24190		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IT99/00355	08/11/1999	09/11/1998
International Patent Classification (IPC) or national classification and IPC C12N15/74		
Applicant G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
01/06/2000	08.02.01
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Grosskopf, R Telephone No. +49 89 2399 8714

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IT99/00355

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-31	as originally filed

Claims, No.:

1-21	as received on	21/11/2000	with letter of	20/11/2000

Drawings, sheets:

1/12-12/12	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-5,9-21
	No:	Claims	6-8
Inventive step (IS)	Yes:	Claims	1-5,13,14,21
	No:	Claims	9-12,15-20

Industrial applicability (IA)	Yes:	Claims 1-21
	No:	Claims

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Ad item IV and V:

The use of the promintron sequence of the rolA gene for the expression of a bacterial gene in root nodules is nowhere disclosed or proposed in the prior art. Therefore, Claims 1 to 5 are novel and inventive.

On the other hand, the use of promoters from the rolA including at least portions of the promintron sequence, both have been proposed and also described (see e.g. D1; EP-A-0204590; the promoter of ORF 10 and D2; The Plant Cell, Vol. 1, (1989), 665-670; the use of the rolA promoter for the expression of the GUS gene).
Thus, Claims 6 to 8 are not novel.
In this context it should be emphasised that Claims 6 to 8 are directed to a DNA but **not** to a bacterial genome or bacterial vector (otherwise they should be drafted accordingly).
But even if e.g. Claim 6 were amended accordingly, it would lack at least an inventive activity over e.g. D2, since the constructs of D2 might be (or even have been) introduced into bacterial vectors for cloning purposes.
It should further be mentioned that the gist of the present application concerns the **use** of a certain sequence (i.e. a method) and the products have no inventive merit per se but at best are suitable means (which have to be adapted accordingly) for carrying out said method. Most of the present product claims do not reflect the invention in a proper manner.

In view of said novelty objection, the remaining Claims 9 to 14 which are directed to different DNA molecules are no longer connected by a common inventive link.

Thus, in order to avoid explicit objections for lack of unity, a new (one) independent claim directed to a DNA molecule should be drafted which corresponds in a better manner to the actual invention (see e.g. present Claim 13).

Novelty, inventive activity and a unitary concept of Claims 15 to 22 are dependent from the novelty and inventive activity of the DNA molecule claim they refer to.

Ad it m VIII:

The reference to a "functional homologous" sequence is unclear in the absence of precisely characterising the function (otherwise any promoter could be covered by said expression).
Moreover, there is no way disclosed how such "functional homologous" (if said function should be specified in any manner) sequences may be isolated.
It might well be that the Applicant had a certain interpretation in mind when drafting the claims. Unless, however, said interpretation becomes part of the claims, this expression remains open for interpretation and, thus, unclear.

The expression "involved in plant hormone synthesis and/or metabolism" are very vague and thus also unclear. Moreover, they are too broad and do not correspond in an adequate manner to the contribution of the present application over the art.
The same, in principle applies for the expressions in Claims 9 and 10

CLAIMS

1. Use of the promintron sequence of the *rolA* gene from *Agrobacterium rhizogenes* as in SEQ ID NO. 1, or of DNA sequences comprising said promintron sequence, or of functional homologous or portion thereof, to induce the expression of a DNA coding sequence, in recombinant bacteria during exponential, post-exponential and stationary phase of growth, and in bacteroids within root nodules, said coding DNA sequence being under the control of said promintron sequence.

2. Use of the promintron sequence according to claim 1 wherein said recombinant bacteria belong to either the *Enterobacteriaceae* or the *Rhizobiaceae* families.

3. Use of the promintron sequence according to claim 2 wherein said recombinant bacteria belonging to either the *Enterobacteriaceae* or the *Rhizobiaceae* families are *E. coli*, *Rhizobia* or *Agrobacteria*.

4. Use of the promintron sequence according to claim 3 wherein said recombinant bacteria are of the *Rhizobia* genus, either within symbiotic root nodules or in a free living status.

5. Use of the promintron sequence according to claim 4 wherein said recombinant bacteria of the *Rhizobia* genus within symbiotic root nodule, are either bacteroids of stage I, II, III, IV, V, or *Rhizobia* present in the apoplastic space, or *Rhizobia* present in the senescence zone, or *Rhizobia* present in the nitrogen fixing zone, or *Rhizobia* present in the invasion zone.

6. A recombinant DNA molecule comprising the promintron sequence according to claim 1, or functional homologous or portion thereof, and covalently linked to the 3′ end of said promintron sequence, a DNA coding sequence, said recombinant DNA molecule being

either harboured by prokaryotic episomal elements, or integrated in a bacterial genome.

7. The recombinant DNA molecule according to claim 6 wherein said DNA coding sequence is either a monocistronic or a polycistronic transcriptional unit.

8. The recombinant DNA molecule according to claim 7 wherein said DNA coding sequence encodes a protein involved in plant hormone auxin synthesis and/or metabolism.

9. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes a protein involved in the synthesis and/or metabolism of the auxin IAA or of the auxin indolethanol.

10. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes the iaaM protein from *P. syringae* subsp. *savastanoi* or an homologous thereof.

11. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes the *tms2* protein from *A. tumefaciens* or an homologous thereof.

12. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes both the *iaaM* and the *tms2* coding regions of claim 10 and 11, respectively.

13. The recombinant DNA molecule according to claim 8 wherein said DNA coding sequence encodes the indolepyruvate decarboxylase from *Enterobacter cloacae* or an homologous thereof.

14. Genetically engineered bacteria comprising the recombinant DNA molecule according to claims from 6 to 13.

15. Use of the recombinant DNA molecule according to claims from 6 to 13 to significantly increase the size of nodules of a plant.

16. Use of the recombinant DNA molecule according to claim 15 wherein said statistically significant increase of the nodule size is of at least 20%.

17. Use of the recombinant DNA molecule according to claims from 6 to 13 to significantly increase the capacity to fix nitrogen of a nodulated plant.

18. Use of the recombinant DNA molecule according to claim 17 wherein said statistically significant increase of the capacity to fix nitrogen is of at least 20%.

19. Use of the recombinant DNA molecule according to claims from 6 to 13 to significantly increase the plant biomass production.

20. Use of the recombinant DNA molecule according to claim 19 wherein said statistically significant increase of the plant biomass production is of at least 10%.

21. Legume plant infected by bacteria harbouring the recombinant DNA molecule according to claims from 6 to 13 and having a significant increase of the size of nodules, and/or of the nodule capacity to fix nitrogen, and/or of the plant biomass, and/or of the ability to fix nitrogen.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ERNSTSEN, A., ET AL.: "Endogenous indoles and the biosynthesis and metabolism of indole-3-acetic acid in cultures of Rhizobium phaseoli" PLANTA, vol. 171, 1987, pages 422-428, XP002099131 cited in the application the whole document	8-22
A	HIRSCH, A.M., ET AL.: "Plant hormones and nodulation: what's the connection?" PLANT MOLECULAR BIOLOGY, vol. 26, 1994, page 5-9 XP002099132 cited in the application the whole document	8-22

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

BANCHETTI, Marina
Ing. Barzano' & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year) 11 July 2000 (11.07.00)	
Applicant's or agent's file reference PCT24190	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/IT99/00355	08 November 1999 (08.11.99)	09 November 1998 (09.11.98)

Applicant
G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,TZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,DM,EE,ES,FI,GB,GD,GE,GH,GM,HR, HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MW,MX,PT,SD,SG,SI, SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:

Pascal Pirlou

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
BANCHETTI, Marina
Ing. Barzano' & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year) 18 May 2000 (18.05.00)	
Applicant's or agent's file reference PCT24190	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/IT99/00355	08 November 1999 (08.11.99)	09 November 1998 (09.11.98)

Applicant
G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,JP,KP,KR,MA,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,GE,GH, GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL, PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   18 May 2000 (18.05.00) under No. WO 00/28051

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra
Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/month/year)	IMPORTANT NOTICE
18 May 2000 (18.05.00)	
Applicant's or agent's file reference	**International application No.**
PCT24190	PCT/IT99/00355

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
BARZANO & ZANARDO Roma s.p.a.
Attn. Banchetti Marina.
26, Via Piemonte
00187 ROMA
ITALY

Applicant's or agent's file reference PCT24190	Date of mailing *(day/month/year)* 29/03/2000
International application No. PCT/IT 99/00355	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
Applicant G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al.	International filing date *(day/month/year)* 08/11/1999

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Sandra De Jong-van Dam

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (c ntinued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
PCT24190		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IT 99/ 00355	08/11/1999	09/11/1998
Applicant		
G.IN.E.ST.R.A. SOCIETA CONSORTILE A.R.L. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. –

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IT 99/00355

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/74 C12N15/31 A01N63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAGRELLI, A., ET AL.: "Splicing of the rolA transcript of agrobacterium rhizognes in Arabidopsis" SCIENCE, vol. 266, 23 December 1994 (1994-12-23), pages 1986-1988, XP002099126 cited in the application	6-8
A	the whole document	1-5,9-22

X √	EP 0 204 590 A (AGRONOMIQUE INST NAT RECH) 10 December 1986 (1986-12-10)	6-8
A	* fig. 2, nts 9659 - 9743 *	1,2

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2000	29/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SCHMÜLLING, T., ET AL.: "Promoters of the rolA, B, and C genes of Agrobacterium rhizogenes are differentially regulated in transgenic plants" THE PLANT CELL, vol. 1, July 1989 (1989-07), pages 665-670, XP002099127 page 669, right-hand column	6,7
X	DEHIO. C., ET AL.: "Stable expression of a single-copy rolA gene in transgenic Arabidopsis thaliana plants allows an exhaustive mutagenic analysis of the transgene-associated phenotype" MOL. GEN. GENET., vol. 241, 1993, pages 359-366, XP002099128 page 360, right-hand column, last paragraph; figure 5	6-8
A	US 5 085 588 A (LONG SHARON R ET AL) 4 February 1992 (1992-02-04) column 2, line 65 - line 68	1-22
A	EP 0 159 779 A (AGRIGENETICS RES ASS) 30 October 1985 (1985-10-30) the whole document	1,2
A	EP 0 130 047 A (AGRIGENETICS RES ASS) 2 January 1985 (1985-01-02) page 10, last paragraph -page 12, paragraph 1	1,2
A	KONDOROSI, A., ET AL.: "Molecular basis of legume-Rhizobium interactions: potentialsfor improving symbiotic nitrogen fixaton" GRASSLAND FOR OUR WORLD,1993, pages 381-384, XP002099129 page 383	8-22
A	COSTACURTA, A., ET AL.: "Synthesis of phytohormones by plant-associated bacteria" CRITICAL REVIEWS IN MICROBIOLOGY, vol. 21, no. 1, 1995, pages 1-18, XP002099130 the whole document	8-22

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International Application No
PCT/IT 99/00355

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ERNSTSEN, A., ET AL.: "Endogenous indoles and the biosynthesis and metabolism of indole-3-acetic acid in cultures of Rhizobium phaseoli" PLANTA, vol. 171, 1987, pages 422-428, XP002099131 cited in the application the whole document	8-22
A	HIRSCH, A.M., ET AL.: "Plant hormones and nodulation: what's the connection?" PLANT MOLECULAR BIOLOGY, vol. 26, 1994, page 5-9 XP002099132 cited in the application the whole document	8-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IT 99/00355

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0204590 A	10-12-1986	US 5182200 A	26-01-1993
		US 5366887 A	22-11-1994
		US 5466792 A	14-11-1995
		US 5824866 A	20-10-1998
		US 5543501 A	06-08-1996
US 5085588 A	04-02-1992	NONE	
EP 0159779 A	30-10-1985	US 4771002 A	13-09-1988
		AT 64756 T	15-07-1991
		AU 581877 B	09-03-1989
		AU 3910285 A	05-09-1985
		JP 60221080 A	05-11-1985
		JP 6315389 A	15-11-1994
		JP 7112433 B	06-12-1995
EP 0130047 A	02-01-1985	NZ 208557 A	29-11-1988
		US 5001061 A	19-03-1991
		US 5008194 A	16-04-1991
		US 5137816 A	11-08-1992
		ZA 8404684 A	27-02-1985